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Disappearance of Radioactive Chromium Tagged Erythrocytes from Tissues and Body Cavities of Rabbits. (21422)

James W. Hollingsworth. (Introduced by W. H. Crosby)

From the Department of Hematology, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D.C.

Observations on the fate of erythrocytes extravasated from their normal vascular channels have been made in the past by direct histological examination of tissues. A method for estimating the fate of extravasated red blood cells was needed in investigations of traumatic wounding that are being undertaken in this institution. Erythrocytes tagged with radioactive sodium chromate have been used successfully in the estimation of erythrocyte lifespan(1), and the present investigations are concerned with the study of Cr^{51}-labelled cells in tissues and body cavities of rabbits. The ideal isotopic tag for these studies would seem to require the following characteristics: 1) labelling of the hemoglobin of the cells without significant tagging of the stroma, and without damage to the cells even in relatively high concentration; 2) firm binding to the hemoglobin with little loss from the cell, and 3) rapid disappearance from the site after lysis of the cells with no appreciable re-tagging of blood constituents. Radioactive sodium chromate appears to fulfill the first two requirements(1). After infusion of Cr^{51} tagged hemoglobin in humans there is no appreciable re-tagging of red cells or plasma, although about 70% of the injected dose remains in the body(1). There is no evidence concerning the disappearance of chromium from the site of cell lysis, but since the isotope is stored in the body, presumably in cells, it seems possible that it might remain in tissue cells or reticuloendothelial cells at the site of local injection and therefore be of no value in this work.

Initial studies, therefore, were designed to investigate the adaptability of the C^{51}-tagged cells to this problem. Further studies were undertaken, after the limits of the method had been defined, to illustrate the use of the technic in determining the fate of erythrocytes in selected extravascular areas.

Materials and methods. Albino rabbits were used in most experiments, but a few animals of mixed breed were included. Blood in acid-citrate-dextrose solution (ACD) was labelled with sodium chromate ($Na_2Cr^{51}O_4$, Abbott) in activities varying from 3-100 μc/ml of blood. After addition of the isotope, the blood was incubated for one hour at room temperature, washed twice with saline, and prepared for injection. External counting of radioactivity was performed with an *in vivo* scintillation counter mounted vertically on a movable side-arm, and duplicate 1 or 2 minute counts were made of each site. Animals were immobilized on an animal board for counting. In most experiments the external counting was performed with the detection head 2 cm from the site to be studied. Pleural and spinal counting were made with the instrument touching the skin over the counting site. Plasma and blood samples were counted in a well-type scintillation counter for sufficient time to allow a random sampling error of less than 2% in most samples. Correction for physical decay of the isotopes was made in all instances.

Results. I. *Studies on adaptability of the Cr^{51} method to study of extravascular clearance of cells.* Ebaugh, Emerson and Ross(1) have demonstrated that about 1% of the chromium tagged to human erythrocytes disappeared by elution each day. The survival curves of the 4 rabbits in Fig. 1A were similar to those obtained by radioiron studies(2) and by sulfhemoglobin labelling(3). Although some elution of Cr^{51} occurred, the correlation of the mean survival time obtained by this method with the mean survival time obtained by other methods was close enough to indicate that there was no large loss of the isotope from the intact cell. After intravenous injection of stroma-free Cr^{51} labelled hemoglobin the decline in radioactivity and of measured plasma hemoglobin agreed closely. There was no evident labelling of plasma proteins, and during these experiments no radioactivity was detected in the washed erythrocytes. When 1 ml of blood was taken from rabbits, labelled with a relatively large amount of Cr^{51}, and reinjected in an intramuscular site, demonstrable radioactivity appeared in both the circulating

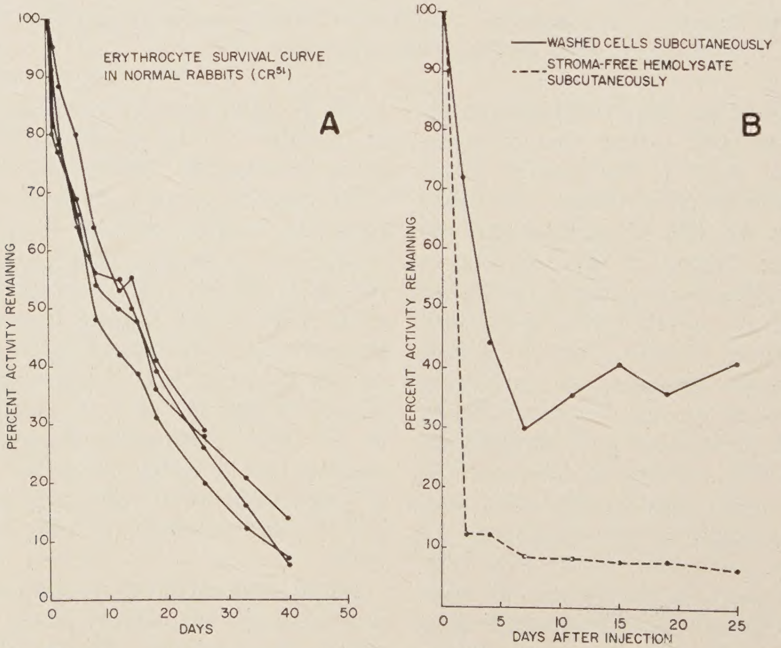

FIG. 1. A. Erythrocyte lifespan of 4 normal rabbits by Cr^{51} method. B. Disappearance of tagged hemoglobin and cells after subcutaneous injection.

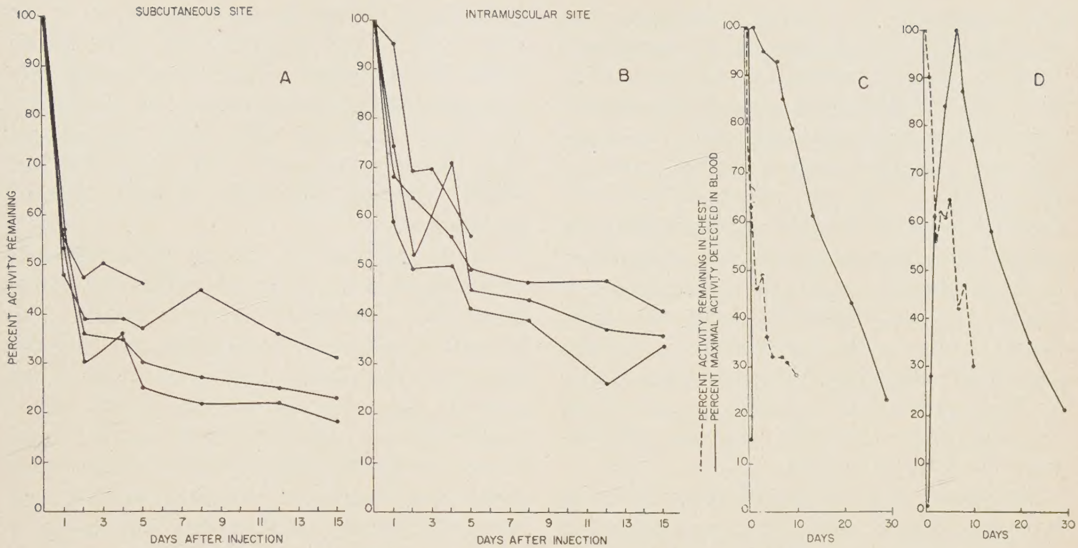

FIG. 2. A. Loss of radioactivity from subcutaneous tissue after injection of tagged cells. B. Loss of radioactivity from intramuscular tissue after injection of tagged cells. C. Loss of radioactivity from the abdomen and appearance in blood after intraperitoneal injection of tagged cells. D. Loss of radioactivity from the chest and appearance in blood after intrapleural injection of tagged cells.

plasma and red cells. The plasma radioactivity fell over a period of a few days. The erythrocyte radioactivity reached a peak in 4-13 days, and then declined at approximately the normal survival rate. Although Cr^{51} did appear in the blood, only 3% of the total injected radioactivity was found in the blood at maximum points. The 100 μc/ml label used in this experiment was 20 times that used in similar subsequent experiments using external counting.

Blood was drawn from rabbits and tagged with approximately 10 μc/ml of blood. After labelling, an aliquot of the sample was hemolyzed by freezing and thawing and the stroma removed by centrifuging in an angle centrifuge. Two subcutaneous areas on the animal's abdomen were chosen, and 0.5 ml of hemoglobin solution was injected at one site and 0.5 ml of tagged cells at the other. The results (Fig. 1B) of surface counting revealed that radioactivity at the site of the injection of the hemoglobin solution had largely disappeared when the second count was performed 2 days after injection. Some radioactivity (less than 10%) remained however, and was unchanged in amount during the entire 25 days of observation. Disappearance of the intact cells proceeded more slowly. About 30% of injected radioactivity remained apparently fixed in the tissues. These experiments indicated that some of the radioactivity remained at the local site after the cells had been hemolyzed. It appeared that this radioactivity was rather permanently fixed and that the fast-falling component actually represented cell lysis.

II. *Studies of clearance of autogenous cells from tissues and body cavities.* Rabbits were injected with 0.5 ml of cells tagged with approximately 5 μc of Cr^{51} per ml subcutaneously over the thorax and intramuscularly in the anterior thigh muscles. The results of the external counting of the injection sites are shown in Fig. 2A and 2B. It can be seen that the baseline of the residual tissue radioactivity was reached in 2-3 days, although activity tended to disappear more gradually from the intramuscular area. Ten ml of blood were tagged with 25 μc of Cr^{51} and the cells reinjected into either the peritoneum or the right pleural cavity. External counting was carried out over the appropriate area, and blood samples were drawn at intervals for measurement of radioactivity.

The cells injected into the peritoneal cavity disappeared very rapidly as indicated by the fall in radioactivity detected by counting over the abdomen, and appeared in the peripheral blood in maximal amount in 1-4 days (Fig. 2C). Radioactivity disappeared more slowly from the pleural cavity than from the abdomen, and maximal activity in the blood did not occur until 4-7 days after injection (Fig. 2D). External counting over the abdomen after the intrapleural injection revealed no radioactivity in the peritoneal cavity, thus excluding any appreciable communication from the right pleural cavity to the peritoneum. Counting over the left chest revealed some radioactivity, but this appeared to be due to scatter from the right chest rather than communication between the 2 pleural spaces.

The results of blood uptake as expressed in the charts (Fig. 2C and 2D) give no indication of the percentage of the blood injected into the pleural or peritoneal cavities which eventually appeared in the circulation. Red cell volume determinations in four normal rabbits revealed a mean value of 17.1 ml/kg body weight, which is almost identical with the mean value obtained by Armin and his co-workers(4) in a large number of rabbits using radioactive phosphorus. Assuming a normal red cell volume of 17.1 ml/kg and knowing the amount of radioactivity injected into the pleural and peritoneal cavities of these animals, the percentage of injected cells reaching the blood stream can be estimated. Since some of the cells are being destroyed on the basis of senescence during this period of absorption into the blood stream the results will always be less than the theoretical 100%.

Estimations of the percentage of tagged cells appearing in the blood stream are shown in Table I. It can be seen that most of the cells given by the intraperitoneal route return to the blood stream, while a small proportion from the pleural cavity escaped to the blood. The survival of the cells which had passed from the body cavities into the circulation appeared identical with that of cells injected intravenously.

TABLE I. Percentage of Injected Cells Absorbed from the Peritoneum and Pleura of 11 Rabbits.

Injection site	% cells absorbed
Peritoneum	91
	63
	60
	56
	62
	69
Pleura	27
	34
	22
	21
	24

Spinal injection of one ml of autogenous cells tagged with 15 μc of radioactive chromium was performed in 2 animals. The amount of radioactivity detected at either the lumbar or the dorsal area varied greatly, apparently depending on the size of the spinal canal of the area after immobilizing the animal in position for counting. Mixing in the spinal canal after injection of cells in the lumbar area occurred promptly in one animal but slowly in another. Although there were daily fluctuations in radioactivity at the 2 counting sites, the total activity of the two sites tended to decrease gradually after 2-3 days, and even after 2 weeks more than half of the activity was still detected in the spinal canal. No attempt was made in this study to differentiate between radioactivity in intact cells or in hemoglobin from lysed cells in the spinal canal.

III. *Studies of human cells in immune and non-immune rabbits.* Human cells, injected subcutaneously and intramuscularly into immunized and normal rabbits, disappeared more rapidly from the animals with circulating antibody against the human cells. Three animals were immunized by weekly intravenous injections of human group-A red cells and 2 animals to human group-B red cells, until saline agglutinins reached a titer of 1:1000 or greater. Injections of Cr^{51}-tagged human cells (0.5 ml) of the appropriate group were then made in subcutaneous and intramuscular sites of 5 immunized rabbits and 6 control rabbits. The differences between the two groups of animals were more clearly defined in the studies of the intramuscular site (Fig. 3) than of the subcutaneous site. It was noted that heterologous human cells disappeared somewhat more slowly from the tissues of the normal animals than did autogenous cells (compare with Fig. 2B). The amount of residual tissue radioactivity varied inversely with the rapidity of cell destruction, so that less residual radio-

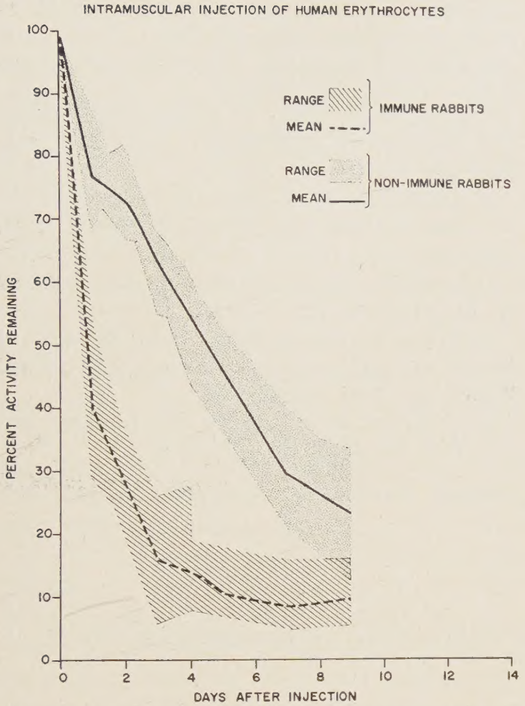

FIG. 3. Disappearance of tagged human cells from 5 immunized rabbits and 6 normal rabbits.

activity was noted in the immunized animals than in the control group.

Discussion. Erythrocytes tagged with radioactive chromium have definite usefulness in studies concerned with the disappearance of red cells from various tissues. The chief disadvantage of this isotopic method is the residual radioactivity remaining in the tissues of the rabbit after cell lysis. When autogenous red cells were injected subcutaneously or intramuscularly, the radioactivity detected by external counting declined rapidly for 2-3 days (presumably due to cell lysis) and then remained essentially unchanged for prolonged periods.

Tagged autogenous cells were rapidly and efficiently reabsorbed from the peritoneum of rabbits and survived normally in the circulation, but intrapleural cells were absorbed more slowly and less completely. Hahn and his co-workers(5) using radioiron-tagged erythrocytes and Jackson(6) using a radioactive iodine compound obtained similar results in studies of peritoneal absorption of erythrocytes. This discrepancy between pleural and peritoneal absorption of cells is difficult to explain, since Allen and Vogt(7) described lymphatic channels capable of absorbing cells on the serous surfaces of both pleura and peritoneum. Our studies indicated little resorption from the spinal canal, although Simmons (8) detected some absorption of radioactive-phosphorus-tagged cells.

Our finding that human erythrocytes disappeared more rapidly from the tissues of immunized animals than from normal animals is probably due to the occurrence, in extracellular fluid, of the potent hemolysin produced in the immunized rabbit. The occurrence of antibody in tissue fluid and lymph has been confirmed repeatedly, and much indirect evidence has been accumulated that soluble antigen disappears rapidly from the tissues of the immunized animals(9). Direct observations of removal of soluble fluorescent antigens from tissues were made by Waksman and Bocking (10). They noted very little change in the amount of antigen in the tissues of immunized and non-immunized rabbits.

The work which we have described deals primarily with the use of Cr^{51}-tagged erythrocytes as a method of investigation of the fate of erythrocytes deposited outside of blood vessels. There may be many specific uses of this method, and the applications reported are primarily those that may be used later in investigations at this institution. The principle of the use of the method lies in the fact that Cr^{51} attaches firmly to the hemoglobin of the intact erythrocyte, stays with the hemoglobin after cell lysis, and is lost only after degradation of hemoglobin occurs. Within this framework, the method may prove of value in studies of erythrocyte lysis or hemoglobin degradation.

Summary. 1. Destruction or removal of erythrocytes from extravascular sites was investigated by the use of cells tagged with radioactive sodium chromate and counted *in vivo*. Limitations and usefulness of the method were studied. 2. Autogenous erythrocytes disappeared from subcutaneous and intramuscular tissues of rabbits in 2-3 days. Less than 3% of the tagged cells appeared in the circulation. 3. After injection of autogenous cells into peritoneal cavity of rabbits, 56-92% of the cells appeared within 1-3 days in the circulating blood and exhibited a normal lifespan. 4. Autogenous cells injected into the right pleural cavity of rabbits appeared in the blood slowly with only 21-34% maximum absorption in 4-7 days. 5. After injection of autogenous cells in the spinal canal of rabbits, the radioactivity disappeared very slowly. 6. Human cells injected into the subcutaneous and intramuscular tissues of rabbits disappeared more rapidly from immunized animals with circulating antibody than from nonimmunized animals.

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In vivo and *In vitro* Conversion of Folic Acid to Citrovorum Factor by Rat.*

(21423)

V. M. Doctor and J. B. Trunnell. (Introduced by J. Awapara)

From Section of Experimental Medicine, University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

The presence in rat liver of an enzyme system converting pteroylglutamic acid (PGA) to citrovorum factor (CF) has been reported (1,2). The conversion of PGA to CF by rat liver slices or homogenate is significantly increased by addition of ascorbic acid(1,2). Welch *et al.*(3) reported that the urinary excretion of CF by rats receiving intraperitoneal injections of PGA was increased by administration of ascorbate or glucoascorbate, but that cysteine and glutathione were ineffective. Addition of homocysteine gives a 50% to 60% increase in the conversion of PGA to CF by chick liver homogenate and under these conditions, glutathione, ascorbic acid and cysteine are without effect(4). Supplementation of chick diet with increasing levels of PGA results in an increased capacity by the chick liver homogenate to convert PGA to CF(5).

In this report the effect of supplementing the rat diet with homocysteine, cysteine and PGA on the urinary excretion of CF is presented as well as studies on PGA to CF conversion by the rat liver homogenate.

Methods. Adult male rats of the Sprague-Dawley strain were placed individually in metabolism cages equipped with metal collecting funnels. Rats were maintained on Purina laboratory chow (Ralston Purina Co.) starting at the weaning stage and throughout the experimental period. The animals were weighed and randomized into four groups of three rats each. Group 1 received the Purina laboratory chow unsupplemented. Group 2, 3 and 4 received a daily supplement of 100 γ PGA by stomach tube. In addition to the latter, groups 3 and 4 were supplemented by daily doses of 15 mg L cysteine and 15 mg DL homocysteine respectively. In a separate experiment 9 rats were randomized into 3 groups of 3 rats each. Group 1 received the Purina laboratory chow unsupplemented. Group 2 and 3 received a daily supplement of 15 mg L cysteine and 15 mg DL homocysteine respectively. Urine collections were started 24 hours after the rats had been maintained on the respective diets and were continued daily for a 2 week test period. The 24 hour urine collections from the 3 rats of the same group were combined and an aliquot of the latter was neutralized, filtered, diluted and assayed for CF. At the end of the 2 week test period the rats from Group 4 were sacrificed and the livers were removed, chilled and immediately tested for PGA to CF conversion according to the previously described procedure(5). The CF content of the urine and the liver samples was determined using the Bacto-CF Assay Medium (Difco Laboratories). *Leuconostoc citrovorum* ATCC 8081† was used as the test organism, and Leucovorin (Lederle) was employed as the standard. The cultures were incubated 48 hours at 37°C and growth was measured by turbidimetric procedure. Since leucovorin has been reported to be half as active as the CF isolated from a horse liver (6), the microbiological assay values were divided by 2 in order to express the results in terms of the naturally occurring CF.

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Results. Administration of 100 γ PGA to rats receiving a practical diet increases by 6 fold the urinary excretion of CF (Table I). This increase is enhanced by supplementing the diet with 15 mg L cysteine or 15 mg DL homocysteine daily by stomach tube. Supplementing the rat diet with 15 mg L cysteine without PGA gives a 50% increase in the urinary excretion of CF. On the other hand the administration of 15 mg DL homocysteine does not markedly influence CF excretion. The rats which received homocysteine (group 4) converted 1.5% to 2% of the administered PGA (100 γ) to CF as measured by the urinary excretion of the factor over a 24 hour period.

TABLE I. Effect of Cysteine and Homocysteine on Urinary Excretion of Citrovorum Factor by Rats Receiving Pteroylglutamic Acid.

Exp.	Supplement to basal, administered by stomach tube daily	Urinary excretion of CF, mγ/rat/day 1st wk	2nd wk
1	0	120	200
	PGA, 100 γ	760	1180
	+ L cysteine, 15 mg	1130	1800
	+ DL homocysteine, 15 mg	1620	2260
2	0	120	220
	L cysteine, 15 mg	190	350
	DL homocysteine, 15 mg	150	170

Studies on PGA to CF conversion by liver homogenates from rats in group 4 are presented in Table II. There is very little conversion of added PGA to CF by the liver homogenate alone. Addition of formate, serine and homocysteine enhances the formation of CF. The stimulatory effect of serine on PGA to CF conversion is further increased by addition of homocysteine. On the other hand homocysteine does not enhance the formate stimulation.

Discussion. The results presented indicate clearly that the *in vivo* conversion of PGA to CF, as measured by the urinary excretion of CF after administration of PGA is enhanced by dietary cysteine and homocysteine. It is interesting to note that the stimulatory effect of homocysteine is significantly higher than that of cysteine. This may be due to a more

TABLE II. Effect of Formate, Serine and Homocysteine on PGA to CF Conversion by Rat Liver Homogenate.

Substances added/flask*	γCF/g liver homogenate
0	2.9
PGA	3.2
+ F†	4.0
+ S	3.9
+ H	4.9
+ F + H	3.8
+ S + H	7.5

* Flask components: 5 ml 20% liver homogenate in 0.08 M sodium potassium phosphate buffer pH 6.3 along with 5 ml of the same buffer and aliquots of solutions containing the following amounts of various substances to make a total volume of 11 ml: PGA, 100 γ; formate, 10 mg; serine, 10 mg; and homocysteine, 5 mg.

† F = formate; S = serine; H = homocysteine.

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† A recent report (Felton, E. A., and Niven, C. F., *J. Bact.*, 1953, v65, 482) concerning a taxonomic study on the culture *Leuconostoc citrovorum* ATCC 8081 suggests that the latter organism is a typical strain of *Pediococcus cerevisiae* as described earlier by Pederson (*Bact. Rev.*, 1949, v13, 225).

direct effect of homocysteine in the PGA to CF converting enzyme system(4). Studies by Sauberlich(7) indicated a 200-fold increase in the urinary CF of rats receiving PGA deficient diet when the latter was supplemented by intraperitoneal injection of 500 γ PGA per day. The administration of ascorbic acid to rats receiving PGA has been reported to enhance the urinary excretion of CF(3). Under these conditions cysteine and glutathione when given by intraperitoneal route are reported to be without significant effects in the amounts tested(3). Extensive studies on the effects of different levels of reducing and sulfhydryl compounds on the CF excretion have not been reported.

Rat liver homogenate (pH 6.3) can convert 4.6% of added PGA to CF in the presence of serine and homocysteine. Chick liver homogenate under identical experimental conditions is reported to convert up to 60% of added PGA to CF(8). It is possible that the present experimental conditions when modified may give a more effective PGA to CF conversion by rat liver homogenate.

Summary. Dietary homocysteine and cysteine enhance the urinary excretion of CF by rats receiving PGA. The stimulatory effect of homocysteine on CF excretion is significantly higher than that of cysteine. *In vitro* conversion of PGA to CF by rat liver homogenate is enhanced by homocysteine, serine and formate. The stimulatory effect of homocysteine on CF synthesis is enhanced by added serine but is inhibited by added formate.

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Studies on Mixed Infections. I. Brucellosis and Q Fever. (21424)

Leonard A. Mika,* Robert J. Goodlow, Joseph Victor,† and Werner Braun.

From the Biological Laboratory, Chemical Corps, Camp Detrick, Frederick, Md., and Department of Bacteriology, George Washington University, School of Medicine, Washington, D. C.

Lennette *et al.*(1), while testing sera of 451 persons with positive *Coxiella burnetii* complement-fixation titers, detected only one person with Brucella agglutinins. These individuals lived in a livestock raising area where an average of 30% positive reactors might have been expected. This observation suggested the existence of interfering effects between *C. burnetii* and Brucella infections and led us to laboratory experiments to be recorded here. It will be shown that an "interference" between *C. burnetii* and *Brucella suis* infections can be demonstrated in guinea pigs and cannot be explained on the basis of conventional immunological responses.

Guinea pigs were exposed either by respiratory route or by parenteral injection and dosage as well as time intervals between exposures to *C. burnetii* and *B. suis* were controlled. Different routes of exposure were selected to determine the influence of natural defense mechanisms of the host. Thus artificial routes, *i.e.* intraperitoneal and subcutaneous infection which may bypass barriers

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* Material presented will form part of forthcoming doctoral dissertation by Leonard A. Mika, George Washington University, Washington, D.C.

† Deceased.

TABLE I. Results of Sublethal Parenteral Exposure of Guinea Pigs to *Brucella suis* and *Coxiella burnetii* as Indicated by Serological Changes.

	Results in exposed animals			Results of reinoculation of spleen homogenates from infected animals		
Organism	Dosage* per animal	Complement fixation test for *C. burnetii*, positive/total animals	% positive	No. of spleens tested	Complement fixation test for *C. burnetii*, positive/total animals	% positive
B. suis	29	0/30	0	5	0/15	0
C. burnetii	1 ID_{50}	16/26	61	29	42/87	48.2
Idem	100 ID_{50}	9/10	90	10	27/30	90.0
B. suis and *C. burnetii*	29 1 ID_{50}	14/29	48.3	30	9/83	10.8
Idem	29 100 ID_{50}	25/27	92.6	27	10/77	13.0

* Represents No. of cells of *B. suis* and/or guinea pig IP ID_{50} of *C. burnetii*.

of resistance, to place animals at a decided disadvantage, were supplemented by respiratory exposures to aerosols containing either or both infectious agents. In addition, both lethal and sublethal doses of *C. burnetii* were employed in these combination studies.

Materials and methods. 1. *Experiments employing sublethal doses of C. burnetii.* A. *Parenteral Infection.* Guinea pigs weighing 300 to 350 g were exposed on the same day a) intraperitoneally to 1 or 100 ID_{50} of *C. burnetii* and b) subcutaneously to 29 cells (approximately 10 ID_{50}) of *B. suis*. Control animals received identical doses of either *C. burnetii* or *B. suis* alone. The dose received by these animals was assayed by viable counts of *B. suis* inoculum and by titration in normal guinea pigs of diluted suspensions of *C. burnetii* inoculum. All exposed animals were bled and sacrificed after 21 days observation and their spleens, regional lymph nodes, and liver samples were cultured for *B. suis*. In addition, homogenates of spleen were prepared and subinoculated into normal guinea pigs for further identification of the presence of *C. burnetii* as indicated by subsequent development of positive complement-fixation titers in test animals. B. *Respiratory Infection.* Guinea pigs were exposed on the same day to aerosols of *C. burnetii* and *B. suis* in the Reyniers Chamber(2). Control animals received identical exposures of either *C. burnetii* or *B. suis* alone. Titration of impinger samples in normal guinea pigs indicated that the dose of *C. burnetii* was 86 guinea pig IP ID_{50} or .012 egg LD_{50} per animal; the inhaled dose of *B. suis* was approximately 5000 cells per animal. Following exposure, clinical studies were carried out at regular intervals and agglutinin, opsonin and complement-fixation titers were determined. Animals were sacrificed at various periods following exposure and all tissues studied in regard to gross and microscopic pathology. Lungs, cervical and bronchial lymph nodes, spleen and liver were cultured for *B. suis*. In addition, spleen homogenates prepared from animals at various periods after aerosol exposure, were injected into normal guinea pigs and blood collected from these secondary test animals 21 days later for determination of complement-fixation titers. 2. *Experiments employing lethal doses of C. burnetii.* A. *Parenteral Infection.* Guinea pigs were exposed subcutaneously to 30 cells of *B. suis* and intraperitoneally to 10^8 guinea pig IP ID_{50} of *C. burnetii*. Infection with *B. suis* preceded infection with *C. burnetii* by either 14 or 28 days. Survivors as well as animals that succumbed, wherever feasible, were tested for presence of *B. suis* and *C. burnetii* as described above. B. *Respiratory Infection.* Guinea pigs were exposed to aerosols of *B. suis*, followed by aerosols of *C. burnetii* either on same day, 6, 11, 14, or 28 days later. The calculated dose of *B. suis* inhaled ranged from 2500 to 5000 cells, the dose of *C. burnetii* from 9.2 x 10^4 to 8.35 x 10^5 guinea pig IP ID_{50} per animal. Control animals were exposed to

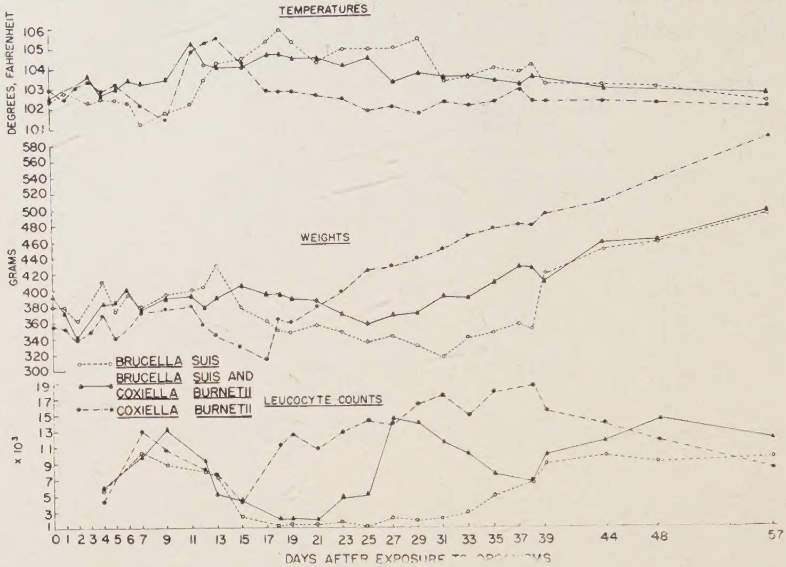

FIG. 1. Temperature, weight and leucocyte determinations of guinea pigs infected with aerosols of *Brucella suis, Coxiella burnetii* and combination of both.

identical doses of *C. burnetii* and *B. suis* alone. Deaths were recorded for 20 days and tissues of animals assayed in the usual manner.

Results. 1. *Experiments employing sublethal doses of C. burnetii.* a. *Parenteral Infection.* Following simultaneous parenteral infection of guinea pigs with 29 cells of *B. suis* and 1 or 100 guinea pig IP ID_{50} of *C. burnetii,* all animals were found infected with *B. suis.* As seen in Table I, 48.3% of the animals that simultaneously had received 1 ID_{50} of *C. burnetii* also had Q fever as indicated by positive complement-fixation tests on the primary exposed combination animals. In group receiving 100 ID_{50} of *C. burnetii* in addition to *B. suis,* 93% of the animals proved infected with *C. burnetii* on the basis of positive complement-fixation titers. However, when spleen homogenates of these "combination animals" were subinoculated into normal guinea pigs complement-fixation titers were obtained only in 10.8% (1 ID_{50}) or 13% (100 ID_{50}) of the secondary test animals. In contrast, control animals receiving the same dose of *C. burnetii* alone, while showing complement-fixation titers in 61% (1 ID_{50}) or 90% (100 ID_{50}) of primary exposed animals, produced positive reactions, with significantly greater frequency in secondary test animals, namely in 48.2% (1 ID_{50}) and 90% (100 ID_{50}), of the animals exposed to spleen homogenates. These results indicate that despite the initial establishment of *C. burnetii* in the primary animals, rickettsiae were not maintained to a sufficient extent in combination with *B. suis* infection to permit comparable serological responses upon subtitration of spleens in normal guinea pigs. Some type of interference between *B. suis* and *C. burnetii* therefore was indicated by results of these initial studies performed on 414 animals.

b. *Respiratory Infection.* Following aerosol exposure to a combination of *B. suis* and sublethal doses of *C. burnetii,* an ameliorative effect of one disease upon the other again was observed. Subtitration of spleen homogenates again indicated a reduced persistence of *C. burnetii* in animals infected with *B. suis* and in addition there was an amelioration of clinical symptoms. Fever, occurring between 11 and 37 days, as well as weight loss and leucopenia were less severe in the combination animals than in control animals infected with *B. suis* alone (see example in Fig. 1). Changes in these clinical symptoms occurred in all 40 combination animals at the same time as in the 32 animals infected with *C. burnetii* alone, but they were less severe, being intermediate

TABLE II. Examples of Serological Changes in Guinea Pigs Infected with Aerosols of *Brucella suis* and/or *Coxiella burnetii*.

Days after exposure	Titers for *C. burnetii* Complement fixation		Titers for *C. burnetii* Opsonin		Titers for *B. suis* Opsonin		Titers for *B. suis* Agglutinin	
				Exposed to *B. suis*				
4	*0	0	0	0	0	0	0	0
10	0	0	0	0	1	0	0	0
14	0	0	0	0	10	10	640	320
17	0	0	0	0	10	10	320	640
21	0	0	0	0	10	10	320	1280
28	0	0	0	0	1	10	1280	640
43	0	0	0	0	100	100	5120	5120
57	0	0	0	0	1000	100	10240	2560
118	0	0	0	0	100	100	2560	5120
				Exposed to *C. burnetii*				
4	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
14	4	128	0	0	0	0	0	0
17	128	128	0	0	0	0	0	0
21	128	128	0	10	0	0	0	0
28	128	256	1	10	0	0	0	0
43	128	256	10	10	0	0	0	0
57	256	128	100	10	0	0	0	0
118	128	16	10	1	10	1	0	0
				Exposed to *B. suis* and *C. burnetii*				
4	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
14	4	0	1	0	1	1	40	640
17	8	64	0	0	1	1	640	640
21	128	64	1	0	1	1	1280	640
28	128	128	10	0	10	10	640	1280
43	128	512	1	10	1000	10	10240	10240
57	128	256	1	10	100	100	5120	2560
118	128	64	10	1	100	10	1280	5120

* For each time period, paired figures represent results obtained from 2 animals with designated serological tests. Values for 54 animals are shown in this table.

between symptoms detected in 64 control guinea pigs infected with either *B. suis* or *C. burnetii* alone. As shown in Table II, agglutinin and opsonin(3) titers for *B. suis* were unaffected by presence of *C. burnetii* and demonstrable at 14 days both in separate and combined infections. Opsonin(4) and complement fixation titers for *C. burnetii* in the combined infection showed no appreciable difference when compared to titers in guinea pigs infected with Q fever alone. Fever persisted in combination animals for about 14 to 24 days, whereas animals infected with *B. suis* alone were febrile for 25 to 35 days. Lymph node changes were less pronounced and lesions less extensive and healed more rapidly in combination animals than in control animals that received the 2 infectious agents separately (see Pathology Section below).

2. *Experiments employing lethal doses of C. burnetii. a. Parenteral infection.* Mortality rates in experiments employing lethal doses of *C. burnetii* also revealed occurrence of an "interference" effect. A total of 180 guinea pigs was used of which 2 groups were exposed to a lethal intraperitoneal dose of *C. burnetii* either 14 or 28 days after subcutaneous *B. suis* infection. As shown in Table III, only 60% of the animals exposed to both infectious agents succumbed when the interval between two exposures was 14 days, whereas 93.3% of the animals died in appropriate control group that had received *C. burnetii* alone. When the interval between primary *B. suis* infection and secondary *C. burnetii* infection was extended to 28 days, only 50% of the animals died in the combination group whereas all animals died in the control group exposed to *C. burnetii* alone. The presence of both *B. suis* and *C. burnetii* in combination animals

TABLE III. Mortality Rates of Guinea Pigs Exposed to *Brucella suis* (Subcutaneously), *Coxiella burnetii* (Intraperitoneally) and Combination of Both.

Days between infection	Organism	Dosage* per animal	Days after *Coxiella burnetii* intraperitoneal exposures 9	10	11	12	13	14	15	16	17	18	20	% deaths
			Accumulative dead/Total animals											
14	*B. suis* (control)	30	—	—	—	—	—	—	—	—	—	—	0/30	0
	B. suis and *C. burnetii*	30 $10^8 ID_{50}$	—	—	—	9/30	12/30	15/30	15/30	16/30	18/30	—	18/30	60
	C. burnetii (control)	$10^8 ID_{50}$	—	2/30	3/30	13/30	19/30	23/30	24/30	25/30	26/30	28/30	28/30	93.3
28	*B. suis*	33	—	—	—	—	—	—	—	—	—	—	0/30	0
	B. suis and *C. burnetii*	33 $10^8 ID_{50}$	—	—	3/30	8/30	11/30	11/30	15/30	—	—	—	15/30	50
	C. burnetii (control)	$10^8 ID_{50}$	1/30	6/30	10/30	20/30	26/30	28/30	30/30	—	—	—	30/30	100

* Represents No. of cells of *B. suis* and/or guinea pig IP ID_{50} of *C. burnetii*.

was indicated by the results of direct tissue cultivation and subtitration of spleen samples.

b. *Respiratory Infection.* Mortality rate of animals exposed to aerosols of *B. suis* and lethal doses of *C. burnetii* on the same day or 6 days later did not differ from that of normal control animals exposed to identical dose of *C. burnetii* (Table IV). However, when the interval between aerosol exposure to *B. suis* and *C. burnetii* was extended to 11 days, the Brucella infected animals displayed marked resistance to Q fever (Table IV). With a dose of 9.2×10^4 guinea pig IP ID_{50} of *C. burnetii,* 4 of 20 Brucella infected animals succumbed to Q fever, as compared with 16 of 20 control animals exposed to the same dose of *C. burnetii.* Similar results were observed when the interval between exposures was 14 and 28 days. A total of 246 animals was used. Again the results of autopsies, tissue cultivation and subinoculation of spleen homogenates, performed on all survivors 20 days following exposure, revealed that all combination animals actually had been infected with both *B. suis* and *C. burnetii.*

3. *Pathological observations.* Gross examination and histological observations indicated a milder disease in combination animals as far as lymph node enlargement and healing of lesions is concerned. Animals exposed to aerosols of *B. suis* and sublethal doses of *C. burnetii* produced less lymph node enlargement and pulmonary consolidation than singly infected control animals. Pulmonary lesions were regressing in combination animals 17 days after exposure and granulomata with definite characteristics of brucellosis (masses of pale-staining mononuclear cells with indistinct outlines, polymorphonuclear cell infiltration and caseous necrosis) appeared in respiratory lymph nodes. Similar changes did not occur in animals infected with *B. suis* alone until at least 21 days after exposure. Lymph node abscesses and fibrosis appeared as early as 4 weeks following exposure in mixed infection, whereas such changes were not noted until 17 weeks in brucellosis alone. Histological studies showed more scarring and necrosis in affected tissues of combination animals at 4 weeks than usually seen in simple brucellosis

TABLE IV. Mortality Rates of Guinea Pigs Exposed to Aerosols of *Brucella suis*, *Coxiella burnetii* and Combination of Both.

Days between infection	Organism	Dosage* per animal	Days after *Coxiella burnetii* respiratory exposure 7	8	9	10	11	12	13	14					% deaths
			Accumulative dead/Total animals												
0	*B. suis* (control)	5306	—	—	—	—	—	—	—	0/10					0
	B. suis and *C. burnetii*	” 4.97 × 10⁵	1/18	3/18	5/18	8/18	10/18	—	—	10/18					55
	C. burnetii (control)	”	—	1/20	3/20	5/20	6/20	7/20	9/20	9/20					45
6	*B. suis* (control)	5306	—	—	—	—	—	—	—	0/10					0
	B. suis and *C. burnetii*	” 4.85 × 10⁵	—	3/20	7/20	7/20	8/20	9/20	—	9/20					45
	C. burnetii (control)	”	—	7/20	10/20	10/20	11/20	12/20	13/20	13/20					65
11	*B. suis* (control)	5306	—	—	—	—	—	—	—	0/20					0
	B. suis and *C. burnetii*	” 9.24 × 10⁴	—	—	—	2/20	4/20	—	—	4/20					20
	C. burnetii (control)	”	—	1/20	5/20	8/20	12/20	15/20	16/20	16/20					80
			Days after *Coxiella burnetii* respiratory exposure 7	8	9	10	11	12	13	14	15	16	17	20	
14	*B. suis* (control)	2509	—	—	—	—	—	—	—	—	—	—	—	0/10	0
	B. suis and *C. burnetii*	” 2.07 × 10⁵	—	—	—	—	—	1/20	1/20	1/20	2/20	—	—	2/20	10
	C. burnetii (control)	”	—	5/20	10/20	12/20	13/20	13/20	13/20	13/20	14/20	—	—	14/20	70

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* Represents No. of cells of *B. suis* and/or guinea pig IP ID_{50} of *C. burnetii*.

at this time. Other pathological findings in combination animals indicated additive effects of both diseases. Scattered areas of minute, red pulmonary consolidation identical with those in Q fever control animals, occurred 4 days after exposure. At this time alveolar and bronchial infiltration of polymorphonuclear leucocytes, but no elementary bodies were noted. Mediastinal lymphatic channels were dilated with polymorphonuclear and mononuclear leucocytes, but lymph nodes were unaltered. After 2 weeks the alveolar exudate was composed mainly of mononuclear cells and fibrin. Perilymphadenitis about tracheal lymph nodes extended widely through the mediastinal fat, and granulomata in the spleen were identical with lesions observed in Q fever control animals. After 21 days the tissue changes in combination animals were usually indistinguishable from those of brucellosis alone, with necrotic granulomata in lymph nodes, spleen, liver, renal pelvis, epididymis and endocardium.

Discussion. These results indicate that guinea pigs exposed to *B. suis* and *C. burnetii* contract both diseases but show an "interference" between these infectious agents. The course of the combined infection was milder and of shorter duration than *B. suis* alone, and guinea pigs infected with *B. suis* proved to be more resistant to lethal doses of *C. burnetii* when 11 days or more had elapsed between the time of exposure to *B. suis* and *C. burnetii.* It is noteworthy that such animals with increased resistance were actually infected with *C. burnetii* as revealed by serological changes in animals subsequently exposed to spleen homogenates which served as indicator of presence of the causative agent in donor animals.

The serological findings in guinea pigs differed from those reported in surveys in humans(1). Agglutinin titers for *B. suis* in guinea pigs with combined infection were similar to those in animals with brucellosis alone. However, preliminary data indicate that following exposure of monkeys (*Macacus rhesus*) to the 2 infectious agents, the serological reactions agree more closely with epidemiological observations of Lennette *et al.*(1): monkeys exposed to both agents developed opsonins against *C. burnetii* and *B. suis,* complement fixing antibodies against *C. burnetii* but no agglutinins against *B. suis.*

Development of increased resistance to Q fever during a specific time interval after infection with *B. suis* might suggest that some immunochemical change may account for these findings. However, blood sera collected from *B. suis* infected guinea pigs when the "interference" effect was demonstrable at 11 days, contained no detectable agglutinins. Such sera when mixed with 10^{-3} and 10^{-8} yolk sac dilutions of *C. burnetii,* and then incubated at 37°C for 12 hours, showed no effect on virulence of the rickettsial organism upon subsequent intraperitoneal injection into guinea pigs.

The milder disease and the enhanced resistance in combined infection, therefore, cannot be explained simply on the basis of demonstrable immunological factors, and it appears unlikely that competition between agents for available substrates can account for the recorded findings, because when administered simultaneously both agents became well established as indicated by presence of both diseases. Moreover, it is unlikely that cellular competition between agents occurs at the time when resistance to lethal doses of *C. burnetii* is high, namely 11 to 28 days after infection with *B. suis.*

These data emphasize the importance of employing healthy animals for experimental use since failure to control interfering spontaneous infections in experimental animals may account for observed discrepancies in susceptibility of laboratory animals to specific infectious agents. Conversely, the extrapolation of data from experiments in which carefully selected healthy animals were used may not always be applicable to problems concerning the natural epidemiology of various diseases. Furthermore, increased resistance to reinfection by one agent cannot be considered conclusive evidence of previous infection by the same or closely related agent as reported by McEwen(5) and others(6) for *B. suis.*

Additional studies have indicated that the results here described may represent only one example of the common occurrence of a hitherto insufficiently recognized phenomenon.

Summary. Guinea pigs exposed simultaneously to *B. suis* and sublethal doses of *C. burnetii* developed both brucellosis and Q fever although animals thus infected showed less severe illness than animals infected with *B. suis* alone. Guinea pigs infected 11 or more days previously with *B. suis* showed a significant resistance to lethal doses of *C. burnetii*. This "interference" cannot be explained on the basis of demonstrable immunological factors.

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Virus Inhibitory Activity of Polyvinylamine.* (21425)

Maurice Green† and Mark A. Stahmann.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Synthetic lysine polypeptides have been shown to inhibit the multiplication of several viruses(1-5). In view of these results, it was of interest to test a non-peptide basic polyelectrolyte for virus inhibitory activity. The results of a survey of the inhibitory action of polyvinylamine, a synthetic basic polyelectrolyte against several animal viruses in the chick embryo are presented here.

Materials and methods. Polyvinylamine hydrochloride‡(6) was dissolved in physiological saline, adjusted to pH 6.3, and autoclaved for 15 minutes at 15 lb pressure. Solutions of lysine polypeptides§(7), protamine sulfate (Krishel Laboratories, Inc., Portland, Oregon), and lysozyme (Armour and Co., Chicago, Ill.) were prepared in a similar manner except that they were sterilized by passing through a Seitz filter disc. *Influenza B virus,* Lee strain, was cultivated in the allantoic sac of 11-day-old embryos. Details on other viruses employed, chick embryo procedures, etc., have been previously described(4,5).

Results. The maximum non-lethal dose per 9-11-day-old chick embryo of polyvinylamine was found to be about 0.2 mg as compared to 1 mg for the lysine polypeptide.

The results of experiments to determine whether polysine and polyvinylamine would inhibit the formation of influenza B virus are presented in Table IA. When polylysine was administered one hour before infection with 20 or 200 infectious doses of influenza B virus, there was a decrease in hemagglutination titer over control of 1.3 log units which reflects a 20-fold reduction in virus growth. Under the same conditions polyvinylamine affected a 0.9 log decrease or a 9-fold reduction in virus growth. Thus, both polylysine and polyvinylamine significantly reduced influenza B virus production. Polylysine however, appears to be more active than polyvinylamine under these conditions.

The inhibition of mumps virus propagation by different quantities of polyvinylamine is presented in Table IB. Significant inhibition of virus growth was obtained when 200 or 40

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† Present address: Research Department, Children's Hospital of Philadelphia, Pa.

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§ The molecular weight of lysine polypeptides in previous papers(4,5) should be corrected as follows: Polylysine of avg. molecular wt 4900 should read 2400 and that of 20,000 should read 16,000.

TABLE I. Inhibition of the Multiplication of Influenza B (IBV) and Mumps Viruses (MV).

	1st inj., 0.1 ml intra-allantoic	2nd inj., 0.1 ml intra-allantoic (1 hr later)	Hemagglutination titer*	Diff. from controls, log
A.	Saline	20 LD$_{50}$ IBV	1024 ± log 0.27	—
	0.4 mg polylysine-HCl†	"	49	1.32
	0.2 " polyvinylamine-HCl	"	101	0.96
	Saline	200 LD$_{50}$ IBV	3090	—
	0.2 mg polylysine-HCl	"	135	1.36
	0.2 " polyvinylamine-HCl	"	407	0.88
B.	Saline	10 LD$_{50}$ MV	65	—
	200 μg polyvinylamine-HCl	"	0	1.81
	Saline	30 LD$_{50}$ MV	102	—
	40 μg polyvinylamine-HCl	"	0	2.01
	10 " "	"	78	0.12
	1 " "	"	65	0.20

* Expressed as reciprocal of geometric mean of hemagglutination titers of allantoic fluids from groups of 3-5 embryos obtained 48 hr and 6 days after infection with influenza B and mumps viruses respectively.

† Polylysine of avg molecular wt 2400.

μg of polyvinylamine were administered per embryo but no inhibition was demonstrated with 10 or 1 μg. Under similar conditions, polylysine of average molecular weight 16,000 inhibited mumps virus multiplication at a concentration of only 1 μg per embryo(5). The molecular weight of the polyvinylamine is not known. Thus, in this case it required only 1/40th as much polylysine as polyvinylamine to demonstrably reduce virus growth.

The data in Table II show that polyvinylamine had a sparing effect for chick embryos infected with 6 LD$_{50}$ of Newcastle disease virus but not against 60 LD$_{50}$. Similar results were obtained with polylysine(4). Polyvinylamine showed only questionable activity when tested against 100 LD$_{50}$ of the Wisconsin strain of infectious bronchitis virus while polylysine gave significant protection against this virus(4). Polyvinylamine was not active when tested against 100 LD$_{50}$ of the Beaudette and Hudson strain of infectious bronchitis virus as was the case with polylysine(4).

TABLE II. The Effect of Polyvinylamine on the Survival of Chick Embryos.

1st inj., 0.1 ml intra-allantoic	2nd inj., 0.1 ml intra-allantoic (1 hr later)	Dead per total*	Avg day of death
	LD$_{50}$ NDV		
Saline	6	13/14	2.8
0.2 mg polyvinylamine-HCl	6	4/10	—
Saline	60	10/10	2.2
0.2 mg polyvinylamine-HCl	60	9/ 9	2.3

* Computed for deaths 6 days after inoculation.

It was of interest to determine whether there was any correlation between the ability of basic polyelectrolytes to inhibit virus formation and their ability to agglutinate red blood cells since both polylysine and protamine have been reported to agglutinate erythrocytes (8,9). Several basic polypeptides and polyvinylamine were therefore titrated with a 1% suspension of chicken erythrocytes to determine their hemagglutination end points. Extremely small quantities of polylysine, protamine, and polyvinylamine (0.05, 0.3, 0.9 μg/ml) would agglutinate the red blood cells, more than 1000 times as much lysozyme was needed. Thus, a rough correlation can be made between the ability of these basic polyelectrolytes to agglutinate erythrocytes and to inhibit virus formation, since polylysine, protamine(4,10), and polyvinylamine all inhibit virus multiplication to some extent while lysozyme(4) had no apparent inhibitory activity against several viruses tested.

Discussion. Polyvinylamine, a synthetic basic polyelectrolyte, was shown to possess virus inhibitory activity somewhat less than that of polylysine. Thus, it seems clear that a polypeptide backbone is not essential for virus inhibition. It may be that the prime requisite for this type of inhibitor is that it be a basic polyelectrolyte. The results with mumps virus revealed that 40 times more polyvinylamine was required to produce inhibitory action comparable to polylysine.

Therapeutically, polylysine has the advantage of being about one-fifth as toxic as polyvinylamine for the chick embryo and probably is metabolized to lysine which is not toxic.

Summary. A comparative study of the virus inhibitory efficacy of the synthetic basic polypeptide, polylysine, and the synthetic basic polymer, polyvinylamine, revealed that both polymers inhibited the production of influenza B virus in the chick embryo. Polyvinylamine had a sparing effect on chick embryos infected with Newcastle disease virus and as little as 40 μg inhibited the multiplication of mumps virus. A comparison with the results of previous studies with polylysine revealed that the polypeptide was more effective than polyvinylamine as a virus inhibitor. It appears probable that the essential feature of this type of virus inhibitor is that it be a basic polyelectrolyte and that a polypeptide backbone may not be essential for activity.

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Ballistocardiography with Electronic Elimination of the Influence of Vibratory Properties of the Body. (21426)

MYRON M. SCHWARZSCHILD. (Introduced by Milton Kissin.)
From the Physics Department, Beth Israel Hospital, New York City.

The ballistocardiogram is a record of movement. In the indirect method, the movement is that of the table; in the direct method, that of the body itself. In either case, the relation between such records and the force exerted upon the body by the heart and vascular system is complicated. If, however, certain simplifying assumptions are made, the relation is expressible as a linear differential equation. These assumptions are that in its elastic behavior tissue obeys Hooke's Law and that frictional forces are due to simple viscosity alone. The linear differential equation expresses a linear relationship among the various quantities: displacement, velocity, acceleration, force and higher derivatives of these quantities.

Such a linear differential equation can be "solved" electronically. Although setting up the equation to reflect accurately the mechanical situation is very difficult, perhaps impossible, certain approximations simplify matters. For the case of direct recording, we assume that approximately the body acts as a rigid mass held in position by damped elastic constraints. Under these conditions, the differential equation is very simple:

$$Ma + Rv + Ed = F$$

where F is the force acting upon the body, M the mass of the body, a the acceleration, v the the velocity, and d the displacement. R and E are coefficients related respectively to the damping viscous resistance and the elastic constants.

It is a relatively simple matter to obtain voltages proportional to a, v and d. This can be done in a variety of ways, for example, with the use of separate transducers for each,

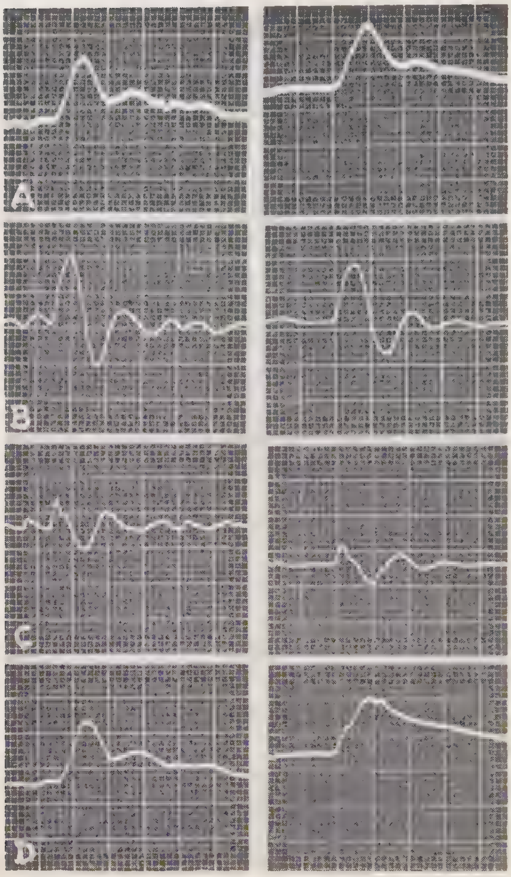

FIG. 1. Records produced by sudden withdrawal of a constant footward force (10 lb). Initial large upward deflection in each record corresponds to headward movement of body due to removal of footward force. *Left column:* A: Displacement. B: Velocity. C: Acceleration. D: Sum in arbitrary proportion before adjustment. *Right column:* A, B and C: Same as on left. D: Sum in correct, adjusted proportions. After-oscillations are absent. Removal of footward force has the same effect as application of a constant headward force. Return of record to base-line in A and D is an artifact due to coupling condensers in amplifier. This return is slow enough to be of no practical importance.

or by picking up velocity with a magnetic pick-up, integrating for *d* and differentiating for *a* by well-known electronic means(1) With 3 such voltages available, they may be combined in the proportions determined by M, R and E and the sum applied to the recording instrument to produce a record of F.

To determine the proper proportions, use is made of the fact that if no force is acting the sum of the 3 left side terms of our equation must be zero. The force can be eliminated for practical purposes by reducing the recorder sensitivity by a large factor (say 10), so that the cardiac forces are ineffective. An external force is then applied to the body by pressure on the shoulders, for example. This force is then suddenly released. Displacement, velocity and acceleration all show after-oscillations. The sum of the 3 in the proper proportions, however, is free of after-oscillations. Thus, the proportions may be found by trial. After having found them, the sensitivity of the recording system is increased to normal. The record of the sum is then the force F. This record is different in many ways from ballistocardiograms taken in the usual way—either displacement, velocity, acceleration or "diagnostic." Fig. 1 illustrates the procedure

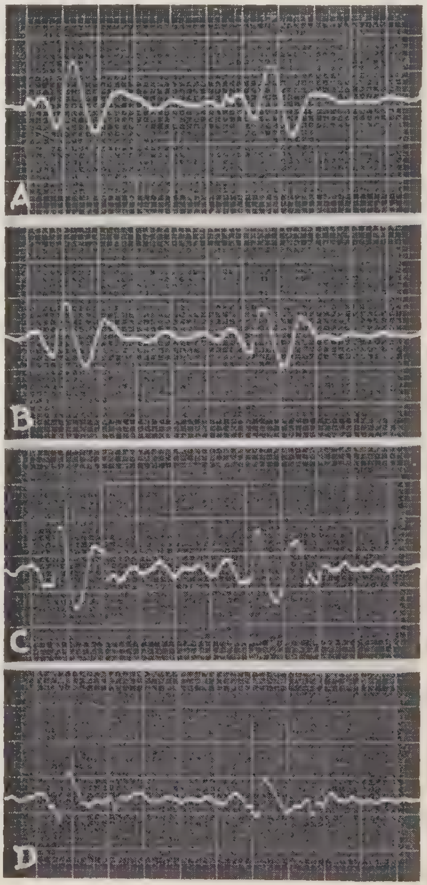

FIG. 2. Record of a normal subject. A: Displacement, with superimposed ECG at low sensitivity. B: Velocity. C: Acceleration. D: Force, the sum in proper proportion of A, B and C.

for finding the correct proportions. Fig. 2 shows the various records obtained in a normal case.

Attempts have been made to accomplish similar elimination of the vibratory properties of the body by purely mechanical means(2). The electronic approach here reported seems to be more direct and simpler to apply. The work is still in a preliminary stage, no clinical studies having yet been completed.

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Fluid Loss Through Epidermis of the Rat and Factors Affecting It.* (21427)

Earl O. Butcher.

From the Departments of Anatomy, College of Dentistry and the Graduate School of Arts and Science, New York University.

A review of the literature shows that there has been little investigation of how much fluid may be lost through the skin of the rat. Burch and Winsor(1), in their investigations of human skin from autopsies, concluded that the corneum was the principal inhibiting layer to the diffusion of water from the body. More recently Blank(2), using similar materials, presents evidence that the major barrier is a very thin layer near the base of the stratum corneum. These investigators, using such material were unable to determine: 1) the loss from living skin; 2) to what extent structural changes in the epidermis would modify fluid loss; and 3) if metabolism and fluid content of the skin which changes at intervals, particularly in the rat(3,4), will alter the fluid loss. Previous investigations on the shedding of the epidermis in the rat showed that applications of unsaturated fatty acids penetrated, altered the epidermis and led to desquamation (5,6). Whether or not the fluid retention properties were affected by the applications was not determined. During these investigations it was frequently noted that linoleic acid was much more effective during the early days (22-24 days) in the resting stage of the hair cycle. Earlier studies(3,4) showed that on these days the fluid content and the metabolism of the skin were also at their minimum. At once one would deduce that the penetration and effectiveness of the acid were related to low metabolism and low fluid content of the skin.

The present study is thus concerned with learning 1) how much fluid may be lost from living skin of the rat; 2) if fluid loss changes with the cyclic activity of the skin; and 3) to what extent the fluid retaining properties of the epidermis are altered by irritants.

Methods. To determine fluid loss through the skin, diffusion chambers similar to those illustrated by Burch and Winsor(1) were used. In each chamber was placed 10 cc normal saline. Rat skin, including panniculus carnosus, from the dorsum and from which hair had been clipped, was placed on the chambers epidermal side out. The diameter of exposed skin was 19 mm. Enough skin was taken from the same rat to cover 2 chambers and these were run simultaneously for comparison. Chambers were inverted and kept on screen in a drying oven at 35°C, humidity 28-32%. Fluid loss was determined by weighing the chambers and skin on analytical balance. A small piece of skin from each rat was also fixed in 10% formalin or Zenker's fluid and histologically prepared. These preparations enabled one to correlate the structural conditions with the fluid retaining property. Because of the large area of skin removed for the two chambers and the biopsy, the rat was of necessity sacrificed. To investigate effect of linoleic acid on fluid loss, it was gently applied morn-

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TABLE I. Fluid Loss ($mg/cm^2/Hr$) through Skin of Rat from Normal Saline (Temp. 35°, Humidity 28-32%).

	Age in days	No. of determinations	Avg fluid loss, $mg/cm^2/hr$	
			1st hr	2nd hr
Normal skin	22	8	1.3 ± .05*	.9 ± .05
	23	8	1.2 ± .03	1.1 ± .04
	25	5	2.0 ± .18	1.1 ± .25
	26	3	2.0 ± .1	1.0 ± .07
	27	8	2.0 ± .1	1.6 ± .1
	28	5	2.7 ± .15	2.3 ± .13
	29	4	2.9 ± .07	2.4 ± .12
	33	4	2.3 ± .07	2.2 ± .04
Linoleic acid on normal animals	22	7	18.2 ±2.0	17.0 ±2.3
	24	3	9.9 ±2.5	8.9 ±3.8
	25	10	8.8 ±1.0	6.7 ±1.6
	27	6	3.8 ± .24	3.5 ± .24
	29	5	3.2 ± .2	3.1 ± .2
Underfed animals	30	8	1.2 ± .07	.6 ± .05
Linoleic acid on underfed animals	30	8	6.7 ± .4	5.2 ± .3

* Stand. error of mean.

ing and evening, to the dorsum of the rat. On the following morning, skin was removed and placed on the two diffusion chambers. Rats were underfed(7) and the fluid loss through their skin determined as well as in underfed animals treated with linoleic acid.

Results. Fluid loss through normal skin. Table I shows extent of fluid loss through the skin at age 22 days or at beginning of resting stage of the hair cycle. At this age the germinativum consists of one or two layers and occasionally granulosa cells are found. The corneum consists of 4 or 5 layers of very flat cells which are dry, hard, and brittle(5). There is less fluid in the skin as previously shown(4), and more is evaporated than is supplied by the underlying tissues. This tends to dry out the corneum which aids in retarding and reducing fluid loss. Since the skin in underfed rats remains in the resting condition, fluid loss of their skin is similar to that in 22-day-old rats. There is a gradual increase in fluid loss as the hair grows. By the 29th day of life fluid loss has increased over 100%. After the 29th day, the loss becomes constant during growing stage of hair cycle. In the 29-day-old rat, the germinativum consists of several layers of cells and a distinct granulosum is present. The presence of a granulosum indicates that the epidermis is more active. This is confirmed by the fact that granules are commonly seen in many prickle cells when activity is caused by an irritant. More of the corneum is retained indicating that it is not as dry in the 22-day-old rat. The fluid content of skin is greater(4) and the ratio of fluid supplied the epidermis by the underlying tissues in respect to evaporation is greater than in a 22-day-old rat and thus a moist corneum exists. A higher metabolism, more fluid in the skin, a more active epidermis and a moist corneum allow greater fluid loss through the skin.

Fluid loss after applications of linoleic acid. The Table shows that great fluid loss occurs in 22-day-old rats after applications of linoleic acid. The loss the first hour was 18.2 mg/sq cm. The corneum was dry and the skin contained less fluid. Under these conditions linoleic acid was able to penetrate and cause great destruction. As the rat increased in age and epidermis became thicker and corneum less brittle, the linoleic acid was much less effective. By 29th day of life the loss is only 3.2 mg the first hour, not much greater than a loss of 2.9 mg through normal skin. Linoleic acid applications also caused the skin of the underfed rat to lose its fluid retention properties. This is expected since the skin of the underfed animal approximates the dry skin of the 22-day-old rat.

Discussion. There is less fluid passage through the skin when the epidermis is thin and inactive and the fluid content and oxygen consumption are low. Inactivity of the epidermis is shown by a lack of granulosum and presence of a brittle corneum. The exact opposite conditions, *i.e.*, presence of a granulosum, a moist corneum and high fluid content of the dermis are conducive to fluid passage through the skin.

As pointed out by Blank(8) if water is lost from the surface of the epidermis at a more rapid rate than it reaches there, the stratum corneum dries out and becomes brittle. When the rats were 22-24 days old, less fluid in the skin resulted in a dry, hard and brittle corneum. This condition of the corneum helps to retard passage of fluid through the skin.

While a thin layer near the base of the corneum may be an important barrier against water loss, as shown by Blank(2) in autopsy material, the present experiments indicate that there are other factors which affect the water loss from the skin.

Summary. Fluid loss through the skin of the rat is the least during its resting stage or when there is a thin inactive epidermis and a dry brittle corneum. Fluid loss is increased as the skin becomes more active and the time of hair growth is approached. Linoleic acid penetrates more during the inactive condition and accentuates water loss.

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Determination of Vitamin B_{12} in Human Serum by a Mutant of *Escherichia coli.* (21428)

N. GROSSOWICZ, J. ARONOVITCH AND M. RACHMILEWITZ
From the Departments of Bacteriology and Medicine "B", Hebrew University, Hadassah Medical School, Jerusalem.

A mutant strain of *E. coli* No. 113-3, isolated and used by Davis and Mingioli(1) for the determination of vit. B_{12} requires either vit. B_{12} or methionine for growth in a mineral medium. The original method was subsequently modified(2,3,4,5,6) with the aim of improving growth in the presence of the vitamin and to increase thereby the accuracy of the test. Burkholder(4) added several amino acids and sodium thioglycolate to the mineral medium and Chiao and Peterson(5) increased the efficacy of the test by shaking the culture tubes. All these modifications proved unsuitable for the determination of vit. B_{12} in serum. Addition of only a few amino acids to the mineral medium in quiescent cultures(4) did not enhance growth appreciably, nor was it sufficient to prevent non-specific stimulation of growth due to substances other than vit. B_{12} present in serum. Shaking causes coagulation of the serum and cannot be used with a turbidimetric procedure.

An assay fulfilling these requirements was developed by adding a methionine-free hydrolysate of casein to the mineral medium of Davis and Mingioli(1).

Methods. The samples of serum were diluted 1:10 with distilled water containing a trace of sodium cyanide (0.5 μg/ml of serum), and were heated at 100°C for 30 minutes to release the bound form of vit. B_{12}(8). The double strength medium (for composition and preparation see Table I) was distributed in 2.5 ml amounts into test tubes, 18 x 150 mm, plugged with cotton and autoclaved at 15 lb. pressure for 10-15 minutes. When cool, ap-

TABLE I. Methionine-Free Casein Hydrolysate Medium.

Potassium diphosphate	3.0 g
Dipotassium phosphate	7.0
Sodium citrate trihydrate	.5
Magnesium sulfate heptahydrate	.1
Ammonium sulphate	.05
Dextrose*	10.0
5% casamino acids, methionine-free†	100 ml
Water to make	500

(adjusted to pH 7.0)

* Dextrose was sterilized separately as a 50% solution and added aseptically to the cool sterile medium.

† Methionine-free casein-hydrolysate was prepared by treating a 10% solution of Casamino-acids "Difco" in N/1 HCl with 0.05M hydrogen peroxide for 24 hr at room temp.(7). Excess of H_2O_2 was then carefully removed by steaming. This procedure also destroys any vit. B_{12} present in the casein hydrolysate.

propriate dilutions of the heated serum samples or graded amounts of a standard solution (sterile) of crystalline vit. B_{12} were added and the volume was made up with sterile distilled water to 5 ml. The bacterial suspension was prepared by removing with a wire loop the growth from a 24 hours agar slant and emulsifying in saline to obtain a barely visible turbidity; the suspension was then diluted 1:10 and one drop was inoculated to each of the test tubes. After incubation for 40-48 hours at 30°C (a temperature of 37°C may be chosen as well) the growth was measured turbidimetrically at mμ 620 (or at 450 mμ) in the Coleman Junior Spectrophotometer.

Results. Fig. 1 represents the growth curves obtained by plotting the optical density of the growth of *E. coli* against the different quantities of crystalline vit. B_{12} used. As evidenced by Fig. 1 the growth in the casamino-acid medium is definitely superior to that of the unsupplemented medium of Davis and Mingioli (1). The vit. B_{12} content of sera of 30 normal subjects measured by this method varied from 200-1000 μμg/ml. These values are higher than those of Mollin and Ross(8) who determined the vit. B_{12} content of human sera by means of *Euglena gracilis*.

Sera of 8 patients with pernicious anemia in relapse were tested and found to contain very small quantities of the vitamin (50-130 μμg/ml. The results are shown in Table II. Patients suffering from nutritional macrocytic anemia were also found to have very small amounts of vit. B_{12} in their serum. In both groups the values of B_{12} increased after injection of crystalline vit. B_{12}.

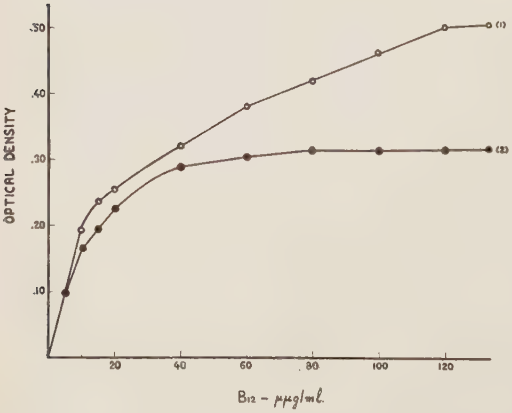

FIG. 1. Growth responses of *E. coli* mutant to different levels of vit. B_{12}. Curve (1). Medium supplemented with methionine-free casein-hydrolysate. Curve (2). Unsupplemented medium (Davis & Mingioli(1)). 9 other experiments gave similar curves.

TABLE II. Vit. B_{12} Content of Sera from Patients with Pernicious Anemia before and after Treatment.

Case No.	Vit. B_{12} in μμg/ml Before treatment	After treatment
1	90	750
2	65	680
3	130, 90*	680
4	75	720
5	80	—
6	50	1050
7	80	600
8	50	500

* Two independent determinations gave different results.

Summary. The vit. B_{12} assay using a mutant strain of *E. coli* was modified by addition of methionine-free casein hydrolysate to a mineral medium. This method gave better growth and allowed a more accurate estimation of the vit. B_{12} than previous methods using the same organism. The assay required only 40-48 hr as compared with 7-9 days when *E. gracilis* is used; the set-up, too, is very simple. The test proved satisfactory for the determination of vit. B_{12} in human serum. The vit. B_{12} content of sera of healthy subjects determined by this method ranged from 200 to 1000 μμg/ml, while sera of patients suffering from pernicious anemia contained only 50-130 μμg/ml.

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Influence of Hypophysis and Adrenal Cortex upon Tissue Mast Cell of the Rat. (21429)

DOUGLAS E. SMITH AND YEVETTE S. LEWIS.*

From the Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.

Finding(1-3) of changes in number and cytology of tissue mast cells after administration of adrenocorticotropic hormone (ACTH) and cortisone has suggested that the pituitary-adrenal system exerts a controlling influence on the mast cell. The present paper describes attempts to explore this possibility further.

Methods. Examinations of the behavior of tissue mast cell were made in whole mounts (4) of skin and mesentery of young adult, male Sprague-Dawley rats (200 g) at various times after adrenalectomy, hypophysectomy and treatment with X-irradiation, ACTH, adrenal cortical extract, desoxycorticosterone acetate or cortisone. Adrenalectomized rats and their controls were allowed to drink only 1% NaCl solution; hypophysectomized animals and their controls were confined to 20% sucrose solution. In addition to regular diet of Rockland checkers, the hypophysectomized animals had access to oranges, lettuce, bread, and ground meat. Tissues were fixed in alcohol and stained with toluidine blue, as previously described(4). Tissues from intact or mock-operated controls were simultaneously studied. The radiation factors were 250 kv, 15 ma, 0.5 mm Cu and 3.0 mm Bakelite filters, 26.7 cm target distance, 1.5 mm Cu half-value layer and 215-225 r per minute. Mast cell counts were made on stained tissues, separate note taken of normal cells and atypical cells, *i.e.* degenerated cells with vacuoles and clumped granules(4,5). On a single slide from each animal, thirty contiguous fields, 0.0676 mm^2 each, were counted at a magnification factor of 264.

Results. The only change in mast cells which could be identified with certainty was an increase in number of cells showing vacuolation and conglomeration of cytoplasmic granules. The alterations were similar to those previously found by us in irradiated animals (4) and by others in aged tissue cultures of mast cells(6), edematous tissue and stimulated urticaria pigmentosa lesions(7), and after treatment with toluidine blue(8), nitrogen mustard(9), ACTH(1) and cortisone(1,2). In none of the present studies were there statistically significant changes in total mast cell number.

Adrenalectomy was without influence upon the mast cell or upon its response to X-irradiation. Groups of 3 to 10 rats were examined at 2, 7, 14, 21, 28 and 36 days after adrenalectomy. Number of cells counted/2.028 mm^2 of skin varied between 404 and 610 in adrenalectomized and 458 and 608 in mock-operated animals. The per cent of abnormal cells was approximately the same in both groups of animals (.2 to 1.2% of all cells). At 2 weeks after adrenalectomy or mock-operation rats were subjected to single, total-body exposures to 600 r. Groups of 3 rats were examined at various intervals after irradiation. Number of cells counted/2.028 mm^2 of skin varied between 328 and 676 in adrenalectomized, irradiated and 339 and 836 in mock-operated, irradiated rats. The per cent of abnormal cells was 10, 33.3, 33.6, 7.0, 3.4 and 2.9 in the adrenalectomized and 10.1, 42.1, 40.0, 5.3, 5.2 and 3.2 in the mock-operated animals at 4 hours, 2, 4, 7, 14 and 22 days after irradiation. (The mast cells of the mesentery responded like those of the skin and, therefore, are not presented.) We interpret these findings to indicate that adrenal cortex of the rat neither regulates the mast cell population nor mediates the response of mast cells to X-irradiation.

Hypophysectomy was attended by a significant, though transient, increase in the number of abnormal cells in the skin, but not in the mesentery (Table I). This suggests that the pituitary is concerned in some way with the maintenance of the integrity of the mast cell in at least one tissue. The results of our studies on adrenalectomized rats indicate that the influence of the pituitary is not mediated through the adrenal cortex. X-irradiation of

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* With assistance of Sally T. Hartig.

TABLE I. Influence of Hypophysectomy upon Mast Cell Number in the Rat.

		Skin				Mesentery			
		Hypophysectomized		Non-operated		Hypophysectomized		Non-operated	
Time (days)	No. of rats[*]	Total No. of cells[†]	% abnormal cells[†]	Total No. of cells	% abnormal cells	Total cells	% abnormal cells	Total cells	% abnormal cells
			I. Hypophysectomized animals						
2[‡]	3	762[§]	.7[§]	729	.0	183	1.6	165	1.8
7	3	712	2.7			260	3.0		
10	3	709	5.4	577	.9	143	2.8	159	3.1
14	5	633	8.8	533	.8	342	.6	258	5.0
16	11	706	14.2	651	.6	152	.7	240	1.1
21	3	707	3.5	673	.7	266	2.6		
28	3	521	3.6	839	1.9	153	2.0	228	.9
36	3	546	2.0	439	1.1	180	.6	296	2.0
			II. X-irradiated[‖] and hypophysectomized animals						
1/6[¶]	6	736	40.0			263	4.6		
2	6	554	56.1	792	28.0	166	26.5	176	18.2
4	6	340	22.9			158	21.5		
7	3	301	7.0			108	21.3		
14	3	475	1.1			92	3.3	161	4.3
22	3	368	1.0	496	2.2	121	1.6		

[*] Per hypophysectomized and non-operated group. [†] Per 2.028 mm^2 of tissue. [‡] Days after operation. [§] Average. [‖] Single total-body exposure to 500 r. [¶] Days after X-irradiation. Hypophysectomized two wk prior to X-irradiation.

the hypophysectomized animals was followed by further increases in the number of abnormal cells (Table I). Results similar to these (Table I) were obtained by us in another experiment in which hypophysectomized rats were exposed to 600 r; in both cases the irradiation effect was added to that caused by hypophysectomy. Thus it appears that the response of the mast cells to X-irradiation is in no way dependent upon an intact pituitary.

The results of administering cortisone and ACTH are presented in Tables II and III. [In the adrenal cortical extract (Armour, 1 cc/rat/day, I.M.) and desoxycorticosterone acetate (5 mg/rat/day, I.M.) studies, counts were made in the skin and mesentery after 1, 2 and 3 days of treatment. Since these preparations induced no changes in the mast cells, the data are not presented.] Neither cortisone nor ACTH produced changes in the mesentery.

TABLE II. Influence of Cortisone upon Mast Cell Number in the Rat.

		Skin		Mesentery	
Treatment	Duration of treatment (days)	Total No. of cells[*]	% abnormal cells[*]	Total No. of cells	% abnormal cells
C,[†] 31 mg/rat/day, subcut.	1	720[‡]	1.8[‡]	200	1.5
	2	703	1.6	325	.6
	3	710	3.5	195	.5
25 " I.M.	1	842	.6	213	.9
	2	920	.5	266	2.3
	3	944	1.2	263	1.1
Control	—	838	2.3	218	.7
C, 10 mg/rat/day, I.M.	2	1063	2.2	175	2.8
	3	1096	3.5	178	.6
5 " "	2	869	4.4	151	.7
	3	896	4.6	232	.5
Control	—	1046	.9	159	.7
C, 10 mg/rat/day, I.M.	3	1017	6.4	207	1.4
5 " "	3	1151	10.1	165	.6
Control	—	1047	.7	159	0

[*] Per 2.028 mm^2 of tissue. [†] C = Cortisone-intact rat. [‡] Avg of 3 animals per treatment group.

TABLE III. Influence of ACTH upon Mast Cell Number in the Rat.

Treatment	Duration of treatment (days)	Skin		Mesentery	
		Total No. of cells*	% abnormal cells*	Total No. of cells	% abnormal cells
ACTH-intact rat					
80 I.U./rat/day, subcut.	1	873†	.7†	229	.3
	2	885	1.8	224	.6
	3	798	.6	219	.5
20 *Idem*	1	560	1.8	215	.9
	2	536	4.8	267	2.2
	3	715	12.1	238	1.3
Control	—	675	.5	194	1.0
ACTH-adrenalectomized rat‡					
20 I.U./rat/day, subcut.	1	434	6.7	259	.4
	2	691	6.1	289	1.3
	3	528	4.4	162	1.9
Control—adrenalectomized	—	459	1.5	240	1.4
ACTH hypophysectomized rat‡					
20 I.U./rat/day, subcut.	1	575	13.2		
Control—hypophysectomized	—	510	4.1		

* Per 2.028 mm^2 of tissue. † Avg of 3 animals/treatment group. ‡ Surgical operations performed 2 wk prior to treatment.

The higher dosages of both hormones were without effect upon the mast cells of the skin. Cortisone in dosages of 5 and 10 mg elicited suggestions of an increase in the number of abnormal mast cells in the skin in one experiment and significant increases in another. ACTH in a dosage of 20 I.U./day was followed by a significant increase in the number of abnormal cells in the skin after 3 days of treatment, with indications of an increase after 2 days. This dosage of ACTH also caused significant increases in abnormal mast cells in the skin of adrenalectomized and hypophysectomized rats.

Although both the hypophysectomy and the ACTH experiments suggest that the anterior pituitary is concerned in some way with maintenance of the integrity of the mast cells of the skin, the present studies do not afford unequivocal information on the mechanism of the action. The positive results with cortisone are suggestive of action through the adrenal cortex: but the bulk of the evidence, (a) lack of effect of adrenal cortical extract, desoxycorticosterone acetate, and adrenalectomy, and (b), effectiveness of ACTH in increasing the number of abnormal mast cells in adrenalectomized rats, strongly indicates that the pituitary influence is not mediated by the adrenal cortex.

The present results of cortisone and ACTH treatment do not agree with prior reports (1,2,3), on rats and other mammals, that these substances cause a decrease in mast cell number. Our data are in accord, however, with recent studies(9,10), which show that total mast cell counts are the same in ACTH or cortisone-treated as in control rats. In 2 of the previous studies(2,3) counts of mast cells were not made and it is possible that erroneous conclusions may have been drawn from subjective impressions concerning mast cell number. We have no explanation for this discrepancy between one of the prior reports on the rat(1) and the present and recent work (9,10), since mast cell counts were made in each instance. It is possible that species differences may account for some of the disagreement. In fact, we have found that, in the hamster(11), both ACTH and cortisone cause large increases in the number of abnormal cells (abnormal cells were 50 to 80% of the total mast cell population) in the mesentery, and small, though significant increases in the number of abnormal mast cells of the cheek pouch and skin. It has been recently reported(12) that cortisone causes a decrease in the number of mast cells in the cheek pouch of the hamster. The increases in the number of abnormal cells found in the present ACTH and cortisone experiments agree with prior claims(1,2,3) but are not in accord with recent reports(9,10) that no such changes occur after these treatments. Actual counts of abnormal mast cells were made only in the present study. It is possible that the failures(9,10) to find increases in abnormal cells may have been due to reliance on subjective impressions as mentioned above.

Recent authors have strongly emphasized (9,10) that actual counts of mast cells must be made in order to evaluate the effect of treatment. Our experience confirms this and in addition indicates that separate account should be taken of abnormal cells. The latter seems especially necessary since the present data show no instances of significant change in total number of mast cells as a result of treatment, but do establish that the number of abnormal mast cells can be increased.

It has been recently claimed(13) that the presence of abnormal mast cells is a consequence of technic. We do not think that the changes ascribed to the experimental procedures employed in the present experiments could be due to such a cause, since fixing, staining, and general handling of all the tissues were carried out under standard conditions (time and temperature of fixing and staining; concentrations of fixative and stain). In each experiment tissues from controls were simultaneously prepared and always contained 1 to 2% abnormal cells. In addition, we have observed increases in the number of abnormal cells in the mesentery of the living rat subjected to ACTH or to X-irradiation(11). Recently it has been reported(14) that all of the mast cells in the cutaneous connective-tissue of the rat are degranulated at 3 weeks after bilateral adrenalectomy. This phenomenon was not apparent at any time after adrenalectomy in the present experiments.

Summary. Mast cell counts were made on skin and mesenteries of rats subjected to adrenalectomy, hypophysectomy, and treatment with ACTH, cortisone, desoxycorticosterone acetate, adrenal cortical extract, and X-irradiation. None of the treatments influenced the total number of mast cells. Adrenalectomy was without influence upon either number of abnormal (vacuolation and conglomeration of cytoplasmic granules) mast cells or response of mast cells to X-irradiation. Hypophysectomy was followed by a transient increase in the number of abnormal mast cells in the skin but not in the mesentery. Hypophysectomy did not alter the response of mast cells to X-irradiation. Cortisone in small dosages increased the number of abnormal cells in the skin of the intact rat. Small amounts of ACTH elicited increases in abnormal cells in the skin of the intact, adrenalectomized, and hypophysectomized rat. Neither ACTH nor cortisone altered the number of abnormal cells in the mesentery.

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Skin Sterols VII. Removal of Δ^7-Cholestenol from Blood of Rats.* (21430)

W. W. Wells and C. A. Baumann.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

The suggestion that Δ^7-cholestenol may be a precursor of cholesterol is based on the finding that the ingestion of the Δ^7 sterol diminishes the rate at which acetate is incorporated into cholesterol(1), and it is supported by the rapidity with which labelled cholesterol accumulates when tritium-labelled Δ^7-cholestenol is fed to rabbits(2). Nevertheless, the distribution of Δ^7-cholestenol is unusual for an active intermediate, since the amounts in skin are often very large(3,4), *e.g.*, up to 54% of the total sterol of rat epidermis(5). Accordingly, the stability of the sterol when injected into the blood stream became of interest.

Experimental. Δ^7-Cholestenol was prepared by the hydrogenation of 7-dehydrocholesterol (Bios) in purified dioxane in the presence of activated Raney nickel at atmospheric pressure(6), and recrystallized from absolute methanol and ethyl acetate: m.p., 122-124°C, no absorption in the ultraviolet. Acetylation in acetic anhydride and pyridine yielded Δ^7-cholestenol acetate which crystallized in long needles from absolute methanol and ethyl acetate, m.p. 118-119°C. Emulsions for injection were prepared by dissolving the sterol in Tween-80 (Atlas Powder Co.) and adding water until the Tween constituted 10 or 20% of the final emulsion. One ml of emulsion was injected intracardially into etherized adult male albino rats of the Sprague-Dawley strain, the amounts of sterol ranging from 5 to 14.7 mg per 200 g rat. Blood samples were removed by heart puncture after suitable intervals. One ml of blood was placed in 1:1 acetone: absolute ethanol and analyzed according to the procedure of Sperry and Webb(7) modified to include the determination of "fast-acting sterols" as Δ^7-cholestenol(5,8). Various pooled tissues were placed in redistilled acetone, refrigerated until analysis, the first step being extraction with acetone in a Soxhlet apparatus.

Results. Disappearance of Δ^7-cholestenol from the blood. The results of many experiments all indicated that injected Δ^7-cholestenol is rapidly removed from the blood, although the rate of removal seemed to depend somewhat upon the amount of the sterol injected. When 5 mg of the Δ^7-cholestenol were injected per 200 g rat, only residual amounts remained in the blood after two hours, but when the dose was 14.7 mg, detectable amounts of the sterol were still present in the blood after 24 hours (Table I). Δ^7-Cholestenol appeared to be associated with the cells immediately after injection, but the amounts in the plasma rose to 47.6% of the total after 2 hours. *Tween* 80 was without effect on the sterols of blood when injected in amounts corresponding to twice those used to emulsify the sterol. The rates of removal of Δ^7-cholestenol from the blood, while rapid, nevertheless proved to be slow enough to permit the use of the sterol in an estimation of blood volume. For this purpose 14.7 mg of Δ^7-cholestenol/200 g rat were injected into the heart as before, and from the amounts of fast-acting sterol found in the whole blood after 2 minutes, the blood appeared to account for 6.47-7.5 cc per 100 g of body weight as compared to an average value of 8.41 cc per 100 g when a modified Evans Blue dye procedure(9) was employed. Recorded values range from 4.3 to 6.7 cc per 100 g(10).

Distribution of injected Δ^7-cholestenol. After suitable intervals of time the Δ^7-cholestenol which had been injected into the blood stream was present in the liver, lung, and spleen, as well as in the blood itself (Table I). The amount in the liver tended to remain constant between the second and 24th hour, while that in the lung and spleen decreased by about one-half. The changes in the other organs were not marked. There was no evidence

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TABLE I. Changes in Sterol Concentration of Rat Tissues after Injection of Δ^7-Cholestenol into Blood.

Form inj. and† time after inj.	Blood				Liver			
	Δ^7-Cholestenol found*		Cholesterol found*		Δ^7-Cholestenol found*		Cholesterol found*	
	Free	Total	Free	Total	Free	Total	Free	Total
	mg %							
0	—	—	86	106	7	16	798	1020
Free Δ^7-Chol., 2 min.	120	120	114	150	67	80	970	1245
” 2 hr	69	70	116	152	140	159	867	1102
'' 24	3.0	3.1	96	127	91	161	1135	1465
'' 48	2.8	3.8	67	90	32	66	675	798
Δ^7-Chol. acetate, 2 min.	—	110	69	99	19	57	768	872
” 2 hr	—	25	60	82	41	106	867	1000
” 24	—	1.2	88	119	21	130	784	958
	Spleen				Lung			
0	31	31	1220	1475	35	38	2070	2160
	—	—	—	—	—	—	—	—
(Free Δ^7-Chol.), 2 hr	1586	1693	1938	2183	695	712	2885	3070
” 24	491	1099	1930	2750	79	133	2323	2500
'' 48	98	308	1345	1765	72	95	2260	2760
Δ^7-Chol. acetate, 2 min.	42	69	762	853	44	286	2690	2695
” 2 hr	69	550	1237	1370	45	219	2510	2510
” 24	72	785	1515	1700	45	180	2320	2320

* For tissues other than blood ''mg %'' means mg of sterol/100 g fat-free dry wt. Each value represents a pool of 3 animals.

† Amount inj. was 14.7 mg of free Δ^7-cholestenol or an equimolar amount of its acetate/200 g rat.

of esterification of Δ^7-cholestenol in the blood itself (Table I) but in the tissues there were increases in the absolute amounts of esterified Δ^7-cholestenol between the second and 24th hours, while the percentages of Δ^7-sterol in the ester form increased markedly: to 55% of the total Δ^7-sterol in the spleen, 43% in the liver, and 40% in the lung. The administration of free Δ^7-cholestenol was also followed by transient increases in the cholesterol content of the tissues, maximum increases being observed between the second and 24th hours after injection (Table II).

Distribution of injected Δ^7-cholestenol acetate. Injected Δ^7-cholestenol acetate, like the free sterol, rapidly left the blood and appeared in the liver, spleen, and lungs, but in lesser amounts than when the free sterol was injected (Table I). The acetate remained unhydrolyzed in the blood and accumulated in the spleen and lungs almost entirely in the ester form; in the liver there were small increases in free Δ^7-sterol as well, but these may have been within the error of the determination. Unlike the results observed when free Δ^7-cholestenol was injected, there were no consistent increases in tissue cholesterol after the injection of Δ^7-cholestenol acetate.

Discussion. Murray and Freeman injected cholesterol emulsions into rats and observed the sterol in the Kupffer cells of the liver and spleen, and in the macrophages of the lungs (11). Friedman and Byers concluded that the liver was the organ chiefly responsible for the removal of injected cholesterol(12), and

TABLE II. Balance: Total Amounts of Δ^7-Cholestenol in Tissues after Injection of the Sterol into the Blood.

Time after inj.	0	2-3 min.	2 hr	24 hr	48 hr
mg of sterol inj.*	0	17.5	18.5	17.5	17.5
	Fast-acting sterol in tissues (mg)				
Blood	0	(17.45)†	(10.53)†	(0.44)†	(0.40)†
Liver	.45	1.45	3.48	3.61	1.91
Spleen	.05	1.21	1.85	.84	.71
Lung	.07		1.54	.80	.17
Skull	1.50	3.83	3.04	2.78	—
Carcass	4.06	6.28	6.80	5.25	—
Skin	48.35	56.95	56.17	51.33	—

* 14.7 mg of Δ^7-cholestenol/200 g rat. (Avg of 3 animals in each group.)

† Based on blood volume calculated from initial determination.

that the reticulo-endothelial system plays a role in the normal treatment of dietary cholesterol(13). The present results show that injected Δ^7-cholestenol emulsions are distributed in the body very much like emulsions of cholesterol itself.

The rapid rate at which Δ^7-cholestenol disappears from the blood stream is consistent with its postulated role as a metabolic intermediate, and the temporary increases in the cholesterol content of the blood and certain other organs that accumulate Δ^7-cholestenol might also be interpreted as compatible with the view that the Δ^7-sterol may be converted into cholesterol.

It is of interest that the free Δ^7-sterol appeared to be more active than Δ^7-cholestenol acetate. The esterification of cholesterol is considered one way by which it is rendered less harmful to the organism, as when cholesterol esters accumulate in the liver after the ingestion of excessive amounts of the sterol. In the present study free Δ^7-cholestenol in the liver, lungs, and spleen was gradually replaced by esterified Δ^7-sterol (up to 60% of the total in spleen) although no comparable de-esterification occurred when Δ^7-cholestenol acetate was injected. Furthermore the presence of Δ^7-cholestenol acetate in the tissues was not followed by any substantial increases in cholesterol such as occurred temporarily after the presence of free Δ^7-cholestenol. Thus, the very large amounts of Δ^7-cholestenol esters in the skin of rodents do not necessarily constitute an obstacle to the postulated active role of Δ^7-cholestenol in the rest of the body, or even in the skin itself, since the esters presumably are less active metabolically, whereas, the amounts of free Δ^7-sterol normally present in skin are relatively small (5).

Summary. 1. Δ^7-Cholestenol or its acetate injected into the blood stream of the rat disappeared rapidly, less than half remaining in blood after 2 hours. At this time 34% of the injected Δ^7-cholestenol was found in the lungs, spleen, and liver; 26% remained in these organs after 24 hours. 2. A substantial portion of the Δ^7-cholestenol remaining was in the ester form. Injected Δ^7-cholestenol acetate disappeared without any corresponding accumulation of free sterol. 3. Administration of free Δ^7-sterol resulted in transient increases in the cholesterol content of blood, liver, lungs, and spleen. No comparable increases followed the injection of Δ^7-cholestenol acetate.

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Heart Lesions in Mice Given Diets Deficient in Vitamins E and K. (21431)

F. I. Dessau, L. Lipchuck and S. Klein. (Introduced by B. L. Hutchings)

From Chemical and Biological Research Section, American Cyanamid Co., Research Division, Lederle Laboratories, Pearl River, N. Y.

Sebrell(1) and co-workers have drawn attention to lesions caused by nutritional deficiencies resulting from addition of sulfonamides to purified diets. Ashburn, Daft, Endicott and Sebrell(2) observed heart lesions in rats after sulfaguanidine feeding. They found necrosis of heart muscle followed by fibrosis and also sclerosis of the coronary arteries. Acute myocarditis with necrosis and hemorrhage in the heart muscle was observed by us in rats kept on a purified diet containing 1% sulfaguanidine. In these experiments with rats strict adherence to a dietary regimen free of Vit. E seemed to be a prerequisite for induction of the lesions. Presence of hemorrhages seemed to be another prerequisite. This suggested an investigation into the role of Vit. K. For this purpose the diet used previously in rats was used in mice in nine experiments which are the subject of this report.

Methods. In these experiments the following diet was used:

Sucrose	70%	Choline chloride	1 g/kg
Alc. extr. casein	20	Sulfaguanidine	10
Corn or cocoanut oil	5	Deltaxine (D 2)	2,000 units/kg
Salts #2	5	Vit. A-acetate	10,000
Vit. B_1	10 mg/kg		
B_6	10		
Riboflavin	10		
Ca pantothenate	20		

This diet was deficient in the following factors: Biotin, Folic Acid, Inositol, Nicotinamide, PABA, Vit. E and Vit. K. During the course of the experiments, notes were taken of body weight and occurrence of subcutaneous hemorrhages. Histological studies were limited to the heart.

Experiments. I and II: Experimental diet with and without sulfaguanidine, with and without Vit. E; groups of 10.

III: Experimental diet with and without Vit. E and 3 different oils as sources of fat (corn oil, cod liver oil, cocoanut oil); groups of 10.

TABLE I. Incidence of Heart Lesions as Affected by Vitamin E.

Exp.	Group	Sulfaguanidine supplement (%)	Vit. E	Incidence of heart lesions (groups of 10)
I	1	1	—	3
	2	1	+	0
	3	0	—	0
	4	0	+	0
II	1	1	+	0
	2	1	—	4

IV: Experimental diet tried out on both weanling and adult mice (exp. I-III, all adults; V-IX, all weanlings); groups of 50.

V: Experimental diet supplemented with PABA, 20 g/kg; Inositol, 1 g/kg; Biotin, 2 mg/kg; Nicotinamide, 10 mg/kg; groups of 50.

VI: Experimental diet supplemented with 10-fold the dose of the 4 vitamins added in Exp. V; groups of 50.

VII, VIII and IX: Experimental diet supplemented with PABA, Inositol, Biotin and Nicotinamide as in Exp. V, with 8 different combinations of deficiencies in Folic Acid, Vit. K and Vit. E; groups of 10 in Exp. VII, of 25 in Exp. VIII and of 50 in Exp. IX.

Results. In Exp. I and II heart lesions were absent in groups which either had not received sulfaguanidine or which had received Vit. E with sulfaguanidine (see Table I).

TABLE II. Incidence of Heart Lesions as Affected by Vit. E and Different Oils, 6 Groups.

Source of fat	Vit. E	Incidence of heart lesions (groups of 10)
Corn oil	—	4
Idem	+	0
Cocoanut oil	—	2
Idem	+	0
Cod liver oil	—	4
Idem	+	0

In Exp. III no significant differences were found between the 3 oils used as source of

TABLE III. Effect of PABA-Inositol-Nicotinamide-Supplement on Incidence of Heart Lesions, 2 Groups.

Supplements	No. of mice examined	Incidence of heart lesions
0	49	17
4	49	16

fat. While the cocoanut-oil group had the lowest incidence of heart lesions (Table II), it also had the most severe.

In Exp. IV acute mortality and hemorrhagic tendencies seemed more in evidence in the group of adult mice. Cardiac lesions were slightly more frequent in the weanlings (17 cases in 42 weanlings, 14 in 50 adults.) This difference would hardly seem significant.

Exp. V showed that 4 of the accessory food factors missing in the experimental diet had no effect on the incidence of heart muscle injury (Table III). In Exp. VI, subcutaneous hemorrhages indicative of Vit. K-deficiency were seen in only 6% of the animals, while present in 48% of the controls receiving the 4 supplements. Correspondingly, heart lesions were found in 15 out of 40 mice in the group without the 4 factors, and in 4 out of 39 which received these in a dose 10 times that given in Exp. V.

Exp. VII, VIII and IX were designed to clarify the role of folic acid and Vit. K in relation to Vit. E in the production of heart muscle injury in sulfaguanidine-fed mice. Combined lack of Vit. E and K was found to be responsible for the occurrence of heart lesions in these experiments, while folic acid played no role. The results of Exp. IX confirmed the results of Exp. VII and VIII (Table IV) in regard to the role of Vit. K.

In all 9 experiments, mice on the experimental diet showed growth retardation, emaciation, atrophy of organs and subcutaneous and internal hemorrhages quite similar to those of rats kept on the same diet. The heart muscle lesions showed the same triad of massive capillary hemorrhages, necrosis of heart muscle fibers and leukocytic infiltration as seen in the sulfaguanidine-fed rats. Histological study of the myocardial lesions failed to demonstrate any difference in the character of the heart muscle lesions in these nine experiments. They were of the same kind wherever Vit. K and E were absent. They were not modified by the absence of any other factor.

In mice which recovered from the acute injury, foci of fibrosis were found in the heart muscle sometimes with calcium deposits. These late lesions resembled those found by Ashburn *et al.*(2) in rats fed diets containing sulfaguanidine. Daft, Endicott, Ashburn and Sebrell(3) while ascribing changes in skeletal muscles to the lack of Vit. E, were uncertain about the factors concerned with the produc-

TABLE IV. Effect of Folic Acid, Vit. E and Vit. K on Incidence of Heart Lesions.

Group	Exp.	Folic acid, .4 mg/kg	Vit. K, 2 mg/kg	Vit. E, 250 mg/kg	No. of mice examined	Mice with heart lesions
A	7	—	—	—	8	3
	8	—	—	—	21	4
B	7	+	—	—	7	1
	8	+	—	—	25	6
	9	+	—	—	50	12
C	7	+	+	—	25	0
	8	+	+	—	25	0
D	7	+	+	+	4	0
	8	+	+	+	24	0
	9	+	+	+	25	0
E	7	—	+	—	5	0
	8	—	+	—	18	0
F	7	—	+	+	5	0
	8	—	+	+	22	0
G	7	—	—	+	7	0
	8	—	—	+	18	0
H	7	+	—	+	9	0
	8	+	—	+	20	0

tion of the injury to the heart muscle. Our experiments appear to be the first on record to implicate Vit. K-deficiency as an etiological factor in a particular form of damage to the heart muscle.

Summary and Conclusion. The occurrence of heart muscle lesions in rats and mice given purified diets supplemented with sulfaguanidine is described. Absence of Vit. K and E is found to produce these lesions in mice.

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Acetaldehyde Utilization by Protein-Depleted Dogs and Rats.* (21432)

W. W. Westerfeld and Dan A. Richert.

From the Department of Biochemistry, State University of New York, Medical College at Syracuse.

A previous report(1) demonstrated that some dogs maintained on a purified diet developed a defect in acetaldehyde metabolism. This defect could be demonstrated by a load test in which acetaldehyde was injected intravenously, and its rate of disappearance from the blood determined. Subsequently, the role of dietary protein and molybdenum in maintaining normal levels of xanthine oxidase in rat tissues has been established(2). Since xanthine oxidase can utilize acetaldehyde as a substrate and since the liver aldehyde oxidase is a similar Mo-containing enzyme(3), the present study was undertaken to see if dietary protein and Mo were related to the defect in acetaldehyde metabolism previously observed in dogs.

Methods. Dogs: Each of 6 normal dogs maintained on chow had a normal rate of acetaldehyde metabolism (less than 6 mg% at the 3 minute point), as determined by the load test previously described(1). The dogs were sacrificed, and the livers were analyzed for Mo(4) and for xanthine oxidase in the presence and absence of methylene blue(5). Three other dogs with normal acetaldehyde disappearance curves were placed on a defined protein-free diet identical with the one previously used(1) except that additional glucose replaced the casein. The acetaldehyde load test was run every 2 weeks and found to be normal throughout the dietary period. After 8 weeks on the protein-free diet, 2 of the dogs were sacrificed for liver analysis, and a liver biopsy was removed from the third dog. The biopsied dog was then fed a modified diet in which 24 g casein replaced an equal weight of glucose, and Na_2MoO_4 was added to give a concentration of 1 mg Mo/kg. After 5 weeks on the 24% casein, Mo-containing diet, the acetaldehyde disappearance curve was still normal, and the dog was sacrificed.

The analytical *results* are shown in Table I. Since the acetaldehyde disappearance curves were normal throughout the protein-free, Mo-poor diet, it is evident that the abnormal curves obtained previously cannot be attributed to a deficiency of protein or Mo. Protein deficiency depleted liver xanthine oxidase to undetectable levels and decreased liver Mo to 37% of its starting value. As reported previously(6) no xanthine oxidase activity could be detected in any of the dog livers in the absence of methylene blue. Refeeding protein and Mo in the diet restored liver Mo and xanthine oxidase to normal levels.

The complete lack of correlation between the rate of acetaldehyde metabolism and the concentration of liver xanthine oxidase or molybdenum suggests that neither xanthine

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TABLE I. Effect of a Protein-Free Diet on Liver Xanthine Oxidase and Mo in Dogs.

Diet	No. dogs	Body wt, kg Start	Body wt, kg End	Acetaldehyde disappearance curve	Liver analyses % solids	Liver analyses µg Mo/g dry wt	Liver analyses X. O. (+M.B.)*
						mean ± S.E.	mean ± S.E.
Normal chow-fed controls	6	—	13.1	Normal	28.7	1.31 ± .17	5.8 ± .8
Protein-free diet 8 wk	3	13.4	10.3	”	27.3	.48 ± .07	0
Protein-free diet 8 wk, then 24% casein + Mo 5 wk	1	13.2	18.1	”	25.7	1.44	7

* Liver xanthine oxidase determined in presence of methylene blue and recorded as net oxygen uptake in mm^3/20 min./flask containing 283 mg fresh tissue.

oxidase nor a similar Mo-containing enzyme is a major factor in acetaldehyde metabolism in the dog. However, such a conclusion must be accepted with caution in view of the previously reported(7,8) lack of correlation between xanthine oxidase levels and uric acid formation, and because of the possibility that an enzyme may normally be present in such excess that a decrease in its concentration is not accompanied by a corresponding decrease in function.

Rats: 50 Male albino rats weighing approximately 200 g were divided into 2 equal groups, one of which was maintained on chow and the other on a zero-protein diet(2). After 4 weeks, rats from each group were weighed, injected intraperitoneally with 5 cc of 4% acetaldehyde per kg body weight. At the end of 3, 5 or 8 minutes the animals were sacrificed and blood samples taken. Each blood sample was analyzed for acetaldehyde; livers were analyzed for xanthine oxidase and molybdenum. At the time of sacrifice the chow-fed rats averaged 298 g and the protein-depleted rats weighed 138 g. By averaging the blood acetaldehyde values found at each of the time intervals, an average acetaldehyde disappearance curve for normal and protein-deficient rats was obtained (Table II). In contrast to the dogs, protein depletion in rats did slow the rate of acetaldehyde metabolism. By plotting the logarithm of the blood acetaldehyde concentration vs time, straight lines were obtained with slopes of 0.172 and 0.148 for the normal and deficient rats respectively. The difference in the extrapolated zero time intercept of 1.81 for the normal and 2.02 for the deficient rats accounts for much of the difference between the 2 curves. If the rate of acetaldehyde metabolism is also constant and exponential during the first 3 minutes, then the lack of a common origin for the 2 extrapolated logarithmic lines must be related to some factor other than metabolic rate. The effect of the diet on the rate of acetaldehyde metabolism, therefore, seems to be relatively small. The effect that was obtained can be attributed to the lack of protein in the diet since feeding a similar purified diet containing 19% casein gave a normal rate of acetaldehyde disappearance in rats(1).

TABLE II. Blood Acetaldehyde Values in Normal and Protein Deficient Rats 3, 5 and 8 Minutes after the I.P. Injection of 5 cc/kg of 4% Acetaldehyde. (Mean ± S.E.)

Min. after inj.	Blood acetaldehyde—mg % 3	5	8
Normals	18.8 ± 2.6	9.4 ± .8	2.7 ± .4
Protein deficient	32.8 ± 2.2	20.1 ± 1.8	6.8 ± .5

Liver xanthine oxidase in the normal chow-fed rats, as determined in the presence of methylene blue, averaged 64 ± 1.3; in the protein-depleted rats liver xanthine oxidase was 4 ± 0.6. These values correspond to 41 and 0 respectively for determination in the absence of methylene blue(5). Liver Mo was 1.97 ± .12 µg/g dry weight for the normal chow-fed rats and 0.57 ± .07 for the protein-depleted rats. The marked effect of protein depletion on liver xanthine oxidase and molybdenum in contrast to the relatively small effect on acetaldehyde metabolism would again suggest that these factors are relatively unrelated in the rat as in the dog.

Summary. A protein-free diet fed to dogs for 8 weeks did not alter the overall rate of acetaldehyde metabolism but did decrease the liver

xanthine oxidase from 6 to zero, and decreased the liver Mo from 1.31 to 0.48 μg/g dry solids. Rats fed a protein-free diet for one month had a somewhat slower rate of removal of acetaldehyde. Liver xanthine oxidase (determined with methylene blue) decreased from the normal chow-fed level of 64 to 4, and liver Mo decreased correspondingly from 1.97 to 0.57. The results suggest that xanthine oxidase and other similar Mo-containing enzymes are not major factors in acetaldehyde metabolism *in vivo*.

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In vitro Method for Cultivating Eastern Equine Encephalomyelitis Virus In Teleost Embryos.* (21433)

MANUEL G. SORET AND MURRAY SANDERS.

From Department of Microbiology, University of Miami, South Miami, Fla.

This communication describes further details of experimental procedures for the propagation of eastern equine encephalomyelitis in *Gambusia sp.* embryos cultivated *in vitro*(1).

Materials and methods. EEE virus, obtained from Dr. Karl Habel, was kept at —30°C as mouse brain suspension. The studies were initiated with infective supernatant fluid from 4th passage of mouse brain virus in Maitland-type cultures of minced chick tissue maintained at room temperature (2). Embryos of *Gambusia*, a viviparous teleost, were obtained from an unclassified species found in waters around Bimini, B.W.I., which resembles *G. manni* Hubbs(3). The technic for obtaining *sterile* embryos is a simple one, and is best carried out with the aid of a binocular eye-loop. Gravid *Gambusia* are brought to sterile room in jar containing sea water or dechlorinated fresh water. An active specimen was chosen, placed in natural prone position, massaged gently with a clean gauze to remove mucus and scales. It is momentarily immersed in merthiolate from tail to pectoral fins and in two washings of 70% alcohol, then dried with *sterile* cotton. The cleansed portion is passed lightly through flame to remove lint or traces of alcohol. With a small scissors, (fish in horizontal position) a cleavage is made between anal opening and anal fin at about a 45° angle, tangentially

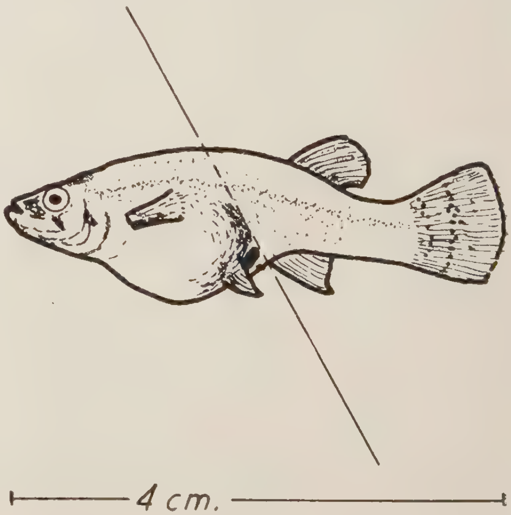

FIG. 1.

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* These studies aided by contract between Office of Naval Research, Department of the Navy, and the University of Miami. The authors wish to express thanks to the Lerner Marine Laboratory for supplying material for this study.

to peritoneal cavity (Fig. 1). If properly carried out, the peritoneum is intact and operating area remains sterile. With a second sterile scissors, an opening is made in the silvery peritoneum and in the ovarian membrane. Gradual pressure on specimen's head, causes the clump of embryos to protrude without rupturing intestines. The entire clump can be removed for examination and cleansing. Embryos are placed in about 10 ml of washing solution (see below), in a dish and as much of the ovarian membrane as is possible is removed with the aid of fine curved forceps or dissecting needles. Embryos are then examined under sterile conditions, at low magnifications (15-30 times), injured or dead embryos being discarded. For practical purposes, embryonic development has been divided into 5 stages described in detail elsewhere(1). Briefly, stage 1 covers period from early fertilized ovum to primitive streak and optic buds are microscopically visible. Stage 2 continues until appearance of epineural melanophores. Stage 3 starting with development of caudal fin, lasts until head protrudes out of extra-embryonic membranes. Stages 4 and 5 represent late embryonic development where swimming movements and gradual absorption of yolk mark completion of embryonic process. Division of embryonic development into 5 stages has been useful in correlating types of embryos with their capacity for supporting virus growth. Several *media were tested* in preliminary fashion and a routine medium selected. Almost all of experimental work has been carried out with this routine medium, which consists of 5 parts of Hanks' salt solution(4), and one part ox serum ultrafiltrate (5) plus 100 units penicillin and 100 γ of streptomycin per ml. The washing solution previously mentioned, into which *Gambusia* embryos are deposited for examination and distribution, is identical with the routine medium except that sodium bicarbonate is omitted to prevent pH rise into alkaline ranges. Various media, tested in short-term experiments included bovine amniotic fluid (6), balanced salt solutions (Simms' or Hanks'), chick embryo ultrafiltrate, physiological salt solution, and Morgan's synthetic medium No. 199(7). Several of combinations of different media appeared to be suitable for embryonic development to varying degrees, but Hanks' solution and serum ultrafiltrate was chosen because of its simplicity and its stabilizing effect on the pH. Combination of Hanks' solution and serum ultrafiltrate has been the only one tested in which the *Gambusia* fertilized ovum developed under *in vitro* conditions to a mature, free-swimming form 22 days after ovum was placed in a roller tube culture. The medium has been employed in 3-5 ml amounts in tubes rotating at speeds of 150-200 r.p.h. The cultures, containing 10-30 *Gambusia* embryos, have been maintained at room temperature (23-25°C). Since superfetation does not occur in *Gambusia* genus(8), embryos in individual clumps were of the same degree of development at given periods. It was therefore possible to determine the stage in which embryos were best as virus substrate by maintaining constant *in vitro* conditions and varying the age of embryos.

Results. The relative suitability of each of the 5 stages of embryo development for virus multiplication was determined by inoculation of cultures; after 4 days an elevation in mouse titer (from LD_{50} 10^{-2} to 10^{-4}) occurred with embryos in stages 1 and 2 only.

In addition to studies of virus activity in *Gambusia* cultures, a continuous line of virus propagation was observed in stage 2 embryos. Because the growth curve of EEE virus in this type of culture was not known, subculture inoculums were occasionally passed at low levels of virus potencies. Furthermore, a number of culture inoculums were derived from quick-frozen specimens stored at —30°C in an effort to evaluate potency before subculturing the virus. Here, too, low potency inoculums were found because stored specimens, consisting of supernatant fluid of cultures without protective colloids such as rabbit serum, decreased one log or more in mouse infectivity before they could be used to inoculate other cultures. Highest mouse potency in this type of culture, appears to be realized 4-7 days post-inoculation.

Three separate culture lines are now under study. The common antecedent for these lines was the same for the first 5 culture gen-

TABLE I. EEE Propagation in *Gambusia* Embryo Cultures.

Culture generation	Potency of culture fluid (mouse LD_{50})		
I		4.9	
II		4.1	
III		4.9	
IV		4.3	
V		4.1	
VI	4.3	5.1	
VII	6.3	3.7	4.7
VIII		5.1	4.9

erations, and the highest mouse LD_{50} for each culture generation is seen in Table I. The usual culture-to-culture inoculum consisted of 0.1 ml of supernatant fluid and in the majority of instances more than 4 mouse titrations were carried out periodically with the supernatant fluid of individual culture generations.

EEE propagation has been observed by quantitative increase of mouse infectivity of supernatant fluids in each culture generation. Furthermore, when the original inoculum of 300 mouse LD_{50} is compared with the highest LD_{50} of $10^{-5.1}$ of the 8th culture generation, the virus has grown in serial cultures after an estimated dilution of original inoculum of $2\text{x}10^{-11}$. Propagation was also seen in 4 experiments when fluid was removed and embryos were washed one or more times. In these instances a second rise in mouse titer was observed. An example of this type of experiment is seen in one of the 7th culture generations (Fig. 2). On the 7th day of incubation, the fluid medium, with a mouse titer of $10^{-4.7}$ was removed and was replaced by fresh medium. In the next 24 hours the process was repeated 3 times and fresh medium added. The culture was returned to the roller tube apparatus and was studied in the usual fashion. The *Gambusia* embryos yielded a second harvest of virus which, in 3 days after multiple washings, reached a level of $10^{-4.1}$ (Fig. 2). In the cell-free control, the virus deteriorated as expected. Bacterial contamination was ruled out by inoculation of supernatant fluids of cultures into bacteriological culture media. Furthermore, absence of latent viruses in embryo tissue and its fluid was determined by frequent injection of mice intracerebrally. On more than 30 occasions supernatant fluid of cultures, free of EEE virus, were injected into mice via intracerebral route. These animals were observed for more than 2 weeks. The same procedure was also carried out with supernatant fluids from ground up, centrifuged

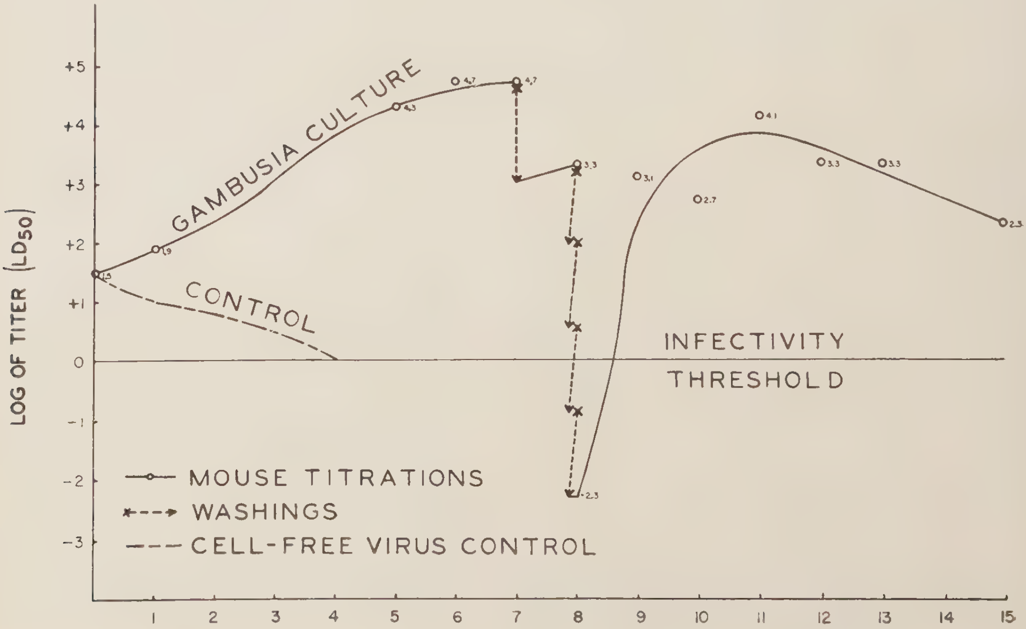

FIG. 2. EEE virus activity in washed *Gambusia* embryos culture (7th subculture).

TABLE II. Specific Nature of Agent Cultivated in *Gambusia* Embryos. Neutralization tests with anti-EEE serums.

Virus source	Serum	Mouse titer LD$_{50}$	Neutralization index
Mouse brain*	C.D.C.†	4.5	3.0
	N.R.S.‡	7.5	
4th subculture	C.D.C.	0.9	3.4
	N.R.S.	4.3	
7th ”	Miami #1	0.5	3.6
	#2	0.0	4.1
	N.R.S.	4.1	
8th ”	Miami #2	0.1	3.6
	N.R.S.	3.7	

* From mouse inoculated with 1st generation *Gambusia* culture.

† Communicable Disease Center, Montgomery, Alabama.

‡ Normal rabbit serum.

embryos. In these tests, aside from occasional traumatic death, no ill effects were observed.

The specific nature of the propagating agent was established by neutralization tests with 3 anti-EEE serums. One antiserum obtained through the courtesy of Dr. Morris Schaeffer of the Communicable Disease Center (CDC), Montgomery, Ala., contained sufficient antibodies to neutralize between 2 and 3 logs of EEE virus. The specificity of the Miami pool of virus was checked, the titer of the CDC serum was confirmed and the virus used to produce 2 lots of anti-EEE rabbit serum. The Miami No. 1 antiserum was identical with the CDC antiserum. The Miami No. 2 neutralized as many as 10,000 EEE mouse intracerebral doses.

The specificity of the mouse infectious agent propagated in *Gambusia* embryo cultures was established by 4 neutralization tests (Table II). The first test was carried out against a mouse brain emulsion, made from sick and dying mice injected intracerebrally with supernatant fluid from the first EEE-infected *Gambusia* culture.† The CDC serum neutralized approximately 1,000 mouse units of this emulsion which was then used for further *Gambusia* embryo cultures, maintained by serial passage. Three additional neutralization tests, with CDC and Miami anti-EEE serums left no doubt that the agent maintained in serial subculture was the virus of eastern equine encephalomyelitis, since neutralization indices varying between 3.4 and 4.1 logs were obtained.

Summary and conclusions. Eastern equine encephalomyelitis virus has been propagated in a new host, *Gambusia sp.* embryos obtained from *field* specimens. A simple method for obtaining sterile embryonic material has been evolved. Standard *in vitro* conditions have been maintenance of embryos in 3-5 ml of 5 parts of Hanks' salt solution plus one part of ox serum-ultrafiltrate, at room temperature (23-25°C) in roller tubes rotating at 150-200 r.p.h. Virus potencies in individual cultures have varied from $10^{-3.7}$ to $10^{-6.3}$ by mouse intracerebral tests, through 8 culture generations. Multiple neutralization tests with the cultured virus have established the specific nature of the propagated agent. Latent mouse viruses in the embryonic material have been ruled out.

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† This culture not included in the genealogy (Table I) because of intervening mouse passage. However, comparison of supernatant potency of culture with the original EEE inoculum, suggested propagation of the virus.

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Catabolism of Ascorbic Acid by Animal Tissues.* (21434)

MIRIAM DE SALEGUI, MORTON A. SCHWARTZ, AND J. N. WILLIAMS, JR.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

In vivo isotope experiments have demonstrated that in the guinea pig, carbon-1 of L-ascorbic acid is rapidly converted to respiratory carbon dioxide and urinary oxalate(1). Little information is available, however, concerning the actual catabolic pathways of this vitamin. It is known that ascorbic acid is reversibly oxidized in animal tissues to dehydroascorbic acid(2-4) and that this compound is in turn irreversibly hydrolyzed to diketogulonic acid(3-5). Diketogulonic acid incubated *in vitro* with rabbit liver slices is subject to enzymatic cleavage(6). However, because of the scant knowledge of the actual mechanism by which ascorbic acid is destroyed in animal tissues, we have studied its catabolism using tissue systems *in vitro*.

Materials and methods. Rat and guinea pig liver preparations used in these experiments were obtained as follows. Adult animals which had been maintained on complete stock rations were decapitated and the livers rapidly removed, weighed and ground in a Potter-Elvehjem glass homogenizer with 3 volumes of chilled Krebs-Ringer phosphate buffer at pH 7.4(7). The homogenates were either used directly or were separated into soluble and insoluble components by centrifugation at 25,000 g for 40 minutes at a temperature of 4°. The supernatant from the centrifugation contained the soluble liver components and will subsequently be referred to as the "extract." The residue, consisting of the insoluble material, was resuspended in buffer to the original volume of the homogenate used. In these studies L-ascorbic acid (Merck) and L-dehydroascorbic acid were used as substrates. The dehydro form of the vitamin was obtained by bromine oxidation of the unneutralized reduced form in distilled water with subsequent aeration to remove the excess bromine. All substrate solutions were prepared and neutralized to pH 7.4 immediately before use. Incubations were carried out in cork-stoppered 25 ml Erlenmeyer flasks shaken in a constant-temperature water bath at 37° for 2 hours. Unless otherwise indicated, 2 mg of neutralized substrate in 1 ml of distilled water were added to an amount of liver homogenate or homogenate fraction equivalent to 0.5 g of fresh tissue and the total volume brought to 6 ml with Krebs-Ringer phosphate buffer. Immediately following the addition of substrate, 1 ml of the reaction mixture was withdrawn and pipetted into 19 ml of mixed acid (5% metaphosphoric acid, 10% glacial acetic) for analysis. A second aliquot was withdrawn at the end of the 2-hour incubation period and treated in the same way. *Ascorbic acid* was determined on the aliquots by the osazone method of Roe and Oesterling(8), as modified by Bolomey and Kemmerer(9) and Bolin and Book(10). The values reported as total ascorbic acid represent the sum of ascorbic, dehydroascorbic and diketogulonic acids. The values reported as per cent destruction represent the difference between total ascorbic acid at the start and end of the incubation period expressed as percentage of the initial value. Except for Table I, in each case where dehydroascorbic acid was used as substrate, the values reported have been corrected for the breakdown of substrate in the absence of liver fraction.

Results. The results of experiments presented in Table I indicate that there is a disappearance of total ascorbic acid in the presence of rat liver homogenate. Since, because of the characteristics of the assay method, this represents changes in the structure of ascorbic acid beyond the diketogulonic acid stage, it is probable that scission of the 6-carbon chain has taken place. As indicated in the footnote to Table I, there was a net increase in the oxidized forms of ascorbic acid when destruc-

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TABLE I. Destruction of Ascorbic and Dehydroascorbic Acids by Whole Liver Homogenates.

System in Krebs-Ringer phosphate at pH 7.4 with 2 mg substrate/0.5 g liver	Destruction of substrate beyond the diketogulonic acid stage (as % of substrate added)	No. of exps.
Ascorbic acid alone	4 ± 1.0†	10
Rat liver homogenate + ascorbic acid	27* ± 1.2	33
Dehydroascorbic acid alone	22 ± 1.6	21
Rat liver homogenate + dehydroascorbic acid	66 ± 3.0	7
Boiled rat liver homogenate + ascorbic acid	2 ± 0.2	7
Guinea pig " " + " "	16 (15–18)‡	4
Boiled guinea pig liver homogenate + ascorbic acid	1 (0–5)	4

* While all of substrate had initially been added in the reduced form, 21% of substrate *remaining* after incubation period had been oxidized to dehydroascorbic or diketogulonic acids.

† Stand. error of the mean.

‡ Figures in parentheses refer to the range of values obtained. This description of variation in results is used when the No. of individual experiments is less than 6.

tion of the reduced ascorbic acid occurred. It can also be seen from Table I that initial substitution of dehydroascorbic acid for ascorbic acid resulted in greater per cent destruction. The path of destruction, therefore, probably involves an initial oxidation of the ascorbic acid to its dehydro form, which is the rate-limiting step.

Experiments in which glutathione was included in the incubation mixture support this conclusion. It was found that the presence of glutathione in equimolar or twice the molar concentration of ascorbic acid had little or no effect on the destruction of either ascorbic acid or dehydroascorbic acid by the homogenate over the 2-hour incubation period. However, when the concentration of glutathione was increased to 10 times that of the ascorbic or dehydroascorbic acid on a molar basis, the destruction was reduced to one-third the original value. Glutathione present in 20 times the molar concentrations of either substrate completely inhibited the destructive activity. This is probably due to a direct effect of glutathione on the substrate since analysis showed that, when the concentration of glutathione was sufficient to give complete protection of dehydroascorbic acid, the latter had been quantitatively converted to its reduced form by the end of the reaction period.

When the homogenate was aged before incubation for a 4-hour period at 37° or for a 7-day period at 5°, its destructive activity on either ascorbic acid or dehydroascorbic acid was not impaired. In fact, the 7-day aging resulted in a slight increase in destruction of both oxidized and reduced forms of the vitamin, indicating that labile, inhibitory (possibly reducing) substances which were destroyed during the aging process were initially present in the fresh homogenate. In contrast, however, it can be seen from Table I that the destructive activity was totally lost when the homogenate was heated for 7 minutes in a boiling water bath before incubation with substrate. A similarly heat-labile destructive activity could be demonstrated in guinea pig liver homogenates (Table I).

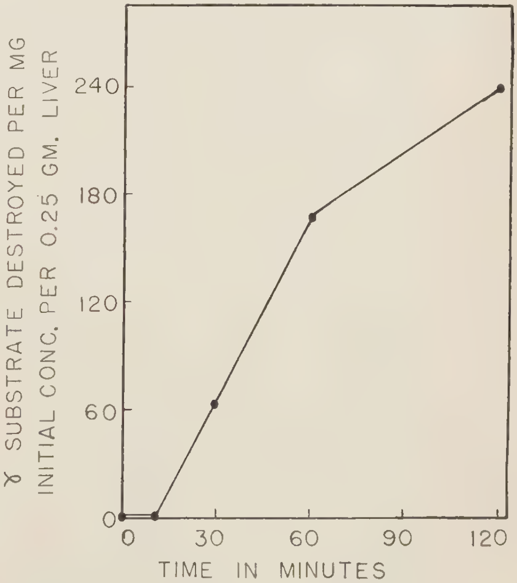

FIG. 1. Destruction of ascorbic acid by whole rat liver homogenates as a function of time.

TABLE II. Localization of Destructive Activity of Rat Liver Homogenates.

System studied	Destruction of substrate beyond the diketogulonic acid stage (as % of substrate added)	No. of exps.
Homogenate + ascorbic acid	27 ± 1.2*	33
Extract + " "	9 ± 1.8	12
Residue + " "	8 ± 1.6	9
Extract + residue + ascorbic acid	36 ± 2.4	8
Residue + boiled extract + ascorbic acid	0 (0–2)†	4
Extract + boiled residue + " "	26 ± 3.9	6
Extract + ashed residue + " "	25 (19–36)	5
Homogenate + dehydroascorbic acid	42 ± 6	6
Extract + residue + dehydroascorbic acid	33	1
Residue + dehydroascorbic acid	5 (0–15)	3
Extract + " "	53 ± 2.7	20
Boiled extract + " "	1 (0–3)	3

* Stand. error of the mean.

† Figures in parentheses refer to the range of values obtained. This description of variation in results is used when the No. of individual experiments is less than 6.

The time curve for the destruction of ascorbic acid by rat liver homogenate showed a pronounced initial lag period (Fig. 1), indicating the existence of at least a 2-step reaction mechanism. This is confirmed by experiments where the homogenate was divided into soluble and insoluble fractions by centrifugation, as described under Methods. Data supporting this conclusion are presented in Table II. When ascorbic acid was incubated with either extract or residue alone, it was not converted to any appreciable extent to degradation products beyond the diketogulonic acid stage. However, when this substrate was incubated with a recombination of these fractions, the destruction was equivalent to that observed with the original homogenate. Furthermore, incubation of ascorbic acid with variously-treated combinations of these fractions indicated that, while both fractions were necessary to achieve destruction of reduced ascorbic acid, only that part of the reaction accomplished by the extract could be inactivated by heating. This is demonstrated by the fact that a combination of boiled or ashed residue with untreated extract caused full destruction of ascorbic acid, while no substrate was destroyed in the presence of boiled extract combined with untreated residue.

Additional information was obtained by replacing ascorbic acid with dehydroascorbic acid as substrate (Table II). Again no destruction was observed in the presence of liver residue alone. However, with dehydroascorbic acid, the extract alone exhibited the same destructive activity as the whole homogenate; and this activity was also found to be heat labile. It was further shown that exhaustive dialysis against distilled water did not reduce the destructive activity of the extract. On the contrary, when compared to the original undialyzed extract that had been stored under similar conditions, it was noted that dialysis resulted in a slightly increased activity (about 10%). When 0.02 mM of 8-hydroxyquinoline was added to either fresh or dialyzed extract in the incubation system, the destructive activity of both preparations was reduced to 48% of its original value. It was also found that when either fresh or dialyzed extract was ashed before incubation, the destruction of dehydroascorbic acid was negligible.

Discussion. From the results of these experiments it can be concluded that an enzyme system responsible for ascorbic acid destruction beyond the diketogulonic acid stage exists in rat and guinea pig liver and can be demonstrated *in vitro* under the appropriate conditions. The complete system which degrades ascorbic acid can be separated into two separate activities, one of which oxidizes ascorbic acid to dehydroascorbic acid and the other of which carries the catabolism of dehydroascorbic acid beyond the diketogulonic acid stage. The former reaction is catalyzed by the insoluble portion of liver and is heat-stable and probably non-enzymatic, as indicated by the fact that the activity for this step remains even after ashing. Once ascorbic acid is converted to its dehydro form, an enzyme system in the soluble fraction of liver accomplishes its breakdown beyond the diketogulonic acid stage. Evidence that the destructive activity of the soluble portion of the liver is enzymatic is as follows: (1) the activity is heat-labile; and (2) the activity is not reduced by exhaustive dialysis. The activity is inhibited, however, by a chelating agent, 8-hydroxyquinoline, indicating that an active metal component is necessary which is bound firmly enough not to be removed by dialysis against water but which can be removed by the chelating agent.

Summary. The catabolism of ascorbic acid in animal tissues has been studied using rat and guinea pig liver homogenates *in vitro*. It has been shown that ascorbic acid is oxidized to its dehydro form by a heat-stable, non-enzymatic factor in the insoluble portion of liver. Dehydroascorbic acid is then further degraded beyond the diketogulonic acid stage by an enzyme system in the soluble portion of the liver.

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Furfural Content of Cigarette Smoke and Its Determination. (21435)

EUGENE W. RICE.* (Introduced by Joseph H. Roe.)
From the Clinical Laboratories, Jewish Hospital Association, Cincinnati, O.

That furfural is a constituent of cigarette smoke has been known for many years. However, a survey of the literature reveals but few references concerning the amount of this aldehyde in cigarette smoke. This is probably due to the impression that furfural is not an important factor in the physiological effects of tobacco smoke, and also to the lack of adequate methods for the determination of the small amount in the complex smoke solutions. Bogen(1) reported values up to 0.2 mg per cigarette in the main stream smoke and amounts up to 0.4 mg in the side stream smoke. However, the technic employed for smoking and smoke collection was not sufficiently standardized and the method used for the determination of furfural(2) was relatively unsatisfactory.

In view of the current speculation as to the role which tobacco smoking, and especially cigarette smoking, may play in the production of cancers of the respiratory tract and other pathology, it is important to re-evaluate present knowledge on tobacco smoke. For this reason, and also to contribute more basic chemical data, a procedure for the determination of furfural in cigarette main stream smoke has been developed. This procedure utilizes the standard technic of experimental smoking (3) and a recent improved photometric method for the determination of furfural(4).

Procedure. Five representative regular length cigarettes of uniform weight are smoked according to the standard technic of smoking and smoke collection described by Bradford, Harlan, and Hanmer(3). For collecting furfural, 5 ml of xylene are placed in the collection flask and 3 ml in each of the 2 bubblers. Puffs of 35 ml volume and 2 seconds duration are taken at intervals of 1 minute until 47 mm of the cigarette is consumed. The number of puffs is recorded and multiplied by 35 to obtain the total volume of smoke. After smoking is completed the smoke is allowed to settle 20 minutes, then the contents of the bubblers and the collection tubes are carefully rinsed with xylene into the smoke collection flask. This solution is transferred to a 25 ml volumetric flask using xylene for rinsing and diluting to the mark. The smoke solution at this stage has a brown coloration which must be removed before the determination of furfural is made. This is accomplished by extraction of the solution with dilute HCl followed by distillation as follows: exactly 1.0 ml of 13% (w/v) HCl is added to the smoke solution, the flask is stoppered tightly and shaken vigorously for a minute. The liquids are allowed to separate and about 10 ml of the xylene layer are transferred to a tube and centrifuged briefly. The xylene is decanted and stirred with a little anhydrous sodium acetate to remove traces of moisture and mineral acid. After centrifugation, 8.0 ml of the smoke solution are pipetted into an all-glass 25 ml-capacity distillation apparatus and distilled almost to dryness directly into a 10 ml volumetric flask. When the distilling flask has cooled to room temperature, 1 ml of xylene is introduced into it. Distillation is resumed and again carried to near-dryness to wash out remaining traces of furfural. This procedure is repeated once more and the distillate is finally diluted to 10 ml with xylene and mixed. The colorless distillate is now ready for the determination of furfural according to the following method of Rice(4). Into one of 3 test tubes or cuvettes is pipetted 1.0 ml of the distillate, into the second 1.0 ml of furfural standard solution (5 μg/ml), and into the third (blank) 1.0 ml of xylene. To each tube is added 5.0 ml of *p*-bromoaniline reagent and immediately mixed by lateral shaking. After 30 minutes standing at room temperature in the dark, the absorbances are determined in the conventional manner with a photoelectric colorimeter at a wavelength of 520 mu, setting the instrument at zero absorbance with the blank solution. The *concentration of furfural* in the smoke is calculated from the absorbance value found for the standard solution and that found for the smoke distillate using the following equations:

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* Present address, Clinical Laboratories, Presbyterian Hospital, Pittsburgh, Pa.

$$\text{(a)}\ \frac{\text{Absorbance of distillate}}{\text{Absorbance of standard}} \times .005 \times 10 \times \frac{25}{8} = \text{mg furfural in xylene phase of smoke sol.}$$

$$\text{(b)}\ \frac{\text{Absorbance of distillate}}{\text{Absorbance of standard}} \times .005 \times .39 = \text{mg furfural in acid phase of smoke sol.}$$

(.39 is coefficient for distribution of furfural between xylene and 13% hydrochloric acid(5).)

$$\text{(c)}\ \frac{\text{(a)} + \text{(b)}}{\text{Total vol. of smoke, L.}} = \text{mg furfural/liter of smoke.}$$

Results. Smoke solutions containing known added amounts of furfural and treated in the prescribed manner gave recoveries of 96 to 98%. The concentration of furfural in the

TABLE I. Concentration of Furfural in Smoke of Various Brands of Cigarettes.

Brand	Total vol of smoke, L	Avg vol of smoke/cigarette, ml	Furfural/L of smoke, mg	Furfural avg/cigarette, mg
Non-filter*				
1	2.24	448	.109	.049
2	2.00	399	.190	.076
3	2.56	511	.027	.014
4	2.45	490	.070	.034
5	2.49	497	.053	.026
6	2.14	427	.178	.076
7	1.75	350	.169	.059
8	2.21	441	.026	.011
		Avg	.103	.043 ± .0236†
Filter*				
9	3.15	630	.0041	.0026
10	2.45	490	.041	.020
11	2.77	553	.064	.035
12	2.45	490	.011	.0053
		Avg	.030	.016 ± .0149†

* Avg wt 5 non-filter cigarettes, 5.32 g (range 5.25-5.63); avg wt 5 filter cigarettes, 5.56 g (range 5.46-5.80).

† Stand. dev. from formula $S_x = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n - 1}}$.

smoke solutions is quite constant, remaining unchanged for at least several weeks.

The concentration of furfural was determined in the smoke from 12 popular brands of cigarettes, with the results shown in Table I. Furfural formation results from combustion of pentosans, pectins, and other related polysaccharides of the tobacco. The amount of furfural in the smoke of non-filter, regular length cigarettes ranged from 0.026 mg to 0.190 mg/liter. This variation may well be largely due to differences in the proportion of air spaces to tobacco shreds within the cigarette, which alter the depth of the combustion zone and the filtering action of the unburned portion of the cigarette. Hence, the looser the tobacco is packed, the more furfural is in the smoke. The marked influence of the physical properties of cigarettes on the composition of the smoke is well known(6).

Table I also shows that the effect of a filter is to remove an appreciable amount of main stream furfural. With an exceptionally heavy and tight filter, the amount of furfural in the smoke may be quite low (cigarettes 9 and 12). With other types of filters, the filtering-out effect may be much less (cigarette 11).

Summary. 1. A method for the determination of furfural in cigarette main stream smoke is presented. The procedure combines the standard technic of experimental smoking and an improved photometric method for the determination of furfural. Concentration of furfural in smoke from 12 popular brands of non-filter and filter regular length cigarettes is given, and some of the factors affecting these data are discussed.

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In situ Indicator Technic, A New Principle in Histochemical Localization Of Enzymes. (21436)

LEE W. WATTENBERG.* (Introduced by J. E. Smadel)

From the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Washington, D. C.

Histochemical procedures now used to localize enzymes in tissue sections depend upon the formation, from reagents in solution, of precipitates which are or can subsequently be made visible. This type of indicator deposition has at least two serious disadvantages. First, choice of substrates is restricted to those whose enzymatic reaction products form insoluble precipitates, a requirement which limits greatly the number of suitable reagents. Second and more important, the precipitation step introduces the serious hazard of false enzyme localization due to diffusion of the reaction products to be visualized. The minute distances separating histological entities emphasize the importance of the many factors which favor diffusion, such as slight solubility of the precipitate and the formation of supersaturated solutions, as well as electrostatic forces. It is therefore not surprising that a search of the literature has failed to produce unequivocal proof that any of the presently known histochemical procedures for the localization of an enzyme is completely accurate. On the contrary, there is evidence that false localization does or can occur(1-7) with many of the procedures described.

The hazard of a diffusion artifact might be circumvented by the use of indicator reagents which are first bound to the constituents of the tissue section and which then, upon incubation in the appropriate substrate solution, would be converted *in situ, i.e.,* in the bound state, by tissue enzymes into a form which is or could be made visible. In the present communication this new principle is applied to the enzyme succinic dehydrogenase, using neotetrazolium chloride (NTC) as the *in situ* indicator.

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* Present Address: Department of Pathology, University of Minnesota School of Medicine, Minneapolis, Minn.

Materials and methods. Preparation of sections. Thin blocks of mouse kidney were frozen in isopentane chilled with liquid nitrogen; and 10 μ sections were cut in a cryostat such as that described by Coons *et al.*(8). The sections were placed on slides without any adhesive coating and dried for about 5 minutes at room temperature. *Procedures for in situ reduction of NTC by succinic dehydrogenase.* The initial step was to bring about binding of the indicator to the tissue constituents prior to its participation in the enzyme reaction by simply placing the slides with the sections for 5 minutes in a freshly filtered solution containing 5 mg per ml NTC in 0.05M phosphate buffer at pH 7.4. The slides were then passed through 4 washings of 0.1M phosphate buffer pH 7.4 to remove the excess NTC. The first wash was for one minute and the succeeding 3 washes were for 3 minutes each. The next step was to initiate the enzyme reaction by incubating the slides at 37°C for 30 minutes in a solution of the substrate which consisted of 0.05M sodium succinate, 0.1M phosphate buffer at pH 7.4, and 0.03M sodium bicarbonate. After incubation, the slides were washed in 0.1M phosphate buffer for about ten minutes and then were mounted in glycerol. *Controls.* There is a small possibility that a positive reaction might not be due to indicator which is reduced *in situ* and held at its original site of binding, but rather to reduction and random deposition of any eluted NTC. This was controlled by incubating sections which had not been pretreated with NTC simultaneously and in the same coplin jar with NTC-pretreated sections. The presence of formazan in the sections not pretreated with NTC could only be due to indicator migration. An additional control for elution was carried out by testing for the presence of indicator in solution following incubation of the sections. This was done by adding sodium hydrosulfite, a reagent which reduces the tetrazol to its corresponding formazan. A third control was included for the purpose of determining the distribution of the tissue-bound neotetrazol. Accordingly, sections which had been passed through the NTC solution and washed were dipped for 30 seconds in a 0.1% solution of sodium hydrosulfite. Finally, for comparative purposes the conventional type of precipitation procedure, basically similar to that of Rutenburg *et al.*(9), was carried out; the succinate reaction mixture used in these experiments was the same as for the *in situ* reduction except that it contained 0.25 mg per ml of NTC.

Results. The *in situ* reduction of NTC by succinic dehydrogenase resulted in the macroscopic appearance of a light purple color in the entire kidney section with the exception of the papilla. Hydrosulfite reduction of tissue bound NTC gave a similar picture except that in this instance the papilla was also colored. In contrast, the NTC-free control sections, which had been incubated in the same jar with the test sections, were devoid of color, and as further indication that NTC had not been eluted in appreciable amounts from the test sections, no formazan formation was observed when hydrosulfite was added to the reaction mixture in which sections with tissue bound NTC had been incubated. Microscopic findings paralleled macroscopic ones and are summarized in Table I. It will be seen that the enzymatic reduction of NTC by succinic dehydrogenase was a selective process as indicated by the presence of formazan in the convoluted tubules and its complete absence in such structures as the collecting tubules, arterial walls, glomeruli, and stroma. In contrast, the non-enzymatic reduction by means of hydrosulfite was of a generalized nature, formazan being found in all of these structures.

The uniformity of binding of NTC to different tissue constituents was controlled by non-enzymatic reduction of the bound indicator with sodium hydrosulfite. This distribution was not uniform. The collecting tubules, glomeruli, and stroma showed less of the indicator than the bulk of the tubular epithelium

TABLE I. Enzymatic and Non-Enzymatic Reduction of Neotetrazolium Chloride as Seen in Sections of Mouse Kidney.

Procedure	Initial solution	Solution for NTC reduction*	Color depth due to formazan† Convoluted tubules	Collecting tubules, glomeruli and stroma	Arterial walls
Enzymatic reduction of NTC *in situ*	NTC, 5 mg/ml, in phosphate buffer	.05M succinate, 30 min.	++	0	0
NTC elution control	Phosphate buffer	*Idem*‡	0	0	0
Non-enzymatic reduction of NTC *in situ*	NTC, 5 mg/ml, in phosphate buffer	.1% sodium hydrosulfite, 30 sec.	++	+	++
Enzymatic reduction of NTC in solution	Phosphate buffer	.05M succinate with NTC, .25 mg/ml 15 min.	++++	0	0

* Sections washed with phosphate buffer prior to being placed in this solution.

† Color depth due to formazan as determined microscopically: 0, colorless; + - ++++, increasing depth of purple color.

‡ Sections incubated simultaneously and in the same vessels with those for enzymatic reduction of NTC *in situ*.

and the arterial walls. On an intracellular level, formazan was found throughout the cytoplasm but little if any was present in the nuclei.

A comparison of the findings of the *in situ* indicator and the precipitation procedures showed little difference in formazan distribution. However, with the *in situ* indicator procedure, cells which reacted positively had a light purple cytoplasm with no visible precipitate (Fig. 1). With the precipitation procedure, a comparable color depth was attained after a 7 minute incubation period. At this time, however, the cytoplasm was red due to partial reduction of neotetrazol to the corresponding monoformazan. Prolongation of incubation resulted in masses of blue diformazan precipitate becoming visible as described by Shelton and Schneider(10). With neither *in situ* indicator nor precipitation procedure was formazan found in nuclei.

Discussion. The present study has shown it possible to demonstrate an enzyme histochemically by *in situ* changes in an indicator previously bound to tissue elements. In the illustrative system employed this *in situ* localization of succinic dehydrogenase in mouse kidney was comparable to that demonstrated by conventional tetrazolium technic. This new method apparently overcomes the serious hazard of diffusion of indicator during its precipitation from solution, a difficulty inherent in older methods. A problem arises, however, which is peculiar to the *in situ* indicator technic and which is illustrated by the failure of NTC to bind to the nuclei, and its failure to be bound uniformly to the various tissue structures. Thus the intensity of the color finally produced will be the result not only of the enzyme activity present but also of the quantity of indicator bound by the tissue component. The necessity for careful controls and interpretation is obvious. Further, it must be stressed that in this study the emphasis has been on elimination of a single source of artifact, namely that due to diffusion of indicator during its precipitation from solution. It is obvious that many other sources of artifact exist which must be controlled in order to achieve accurate microscopic histochemical localization of enzymes. However, since this rapidly expanding field of histochemistry is based on an increasing group of technics which have in common the undesirable feature of precipitation of reagents from solution, alternate principles of indicator deposition are desirable. It is worthy to consider that other substrates for specific enzymes might be developed which would combine chemically with ubiquitously distributed reactive groups in the

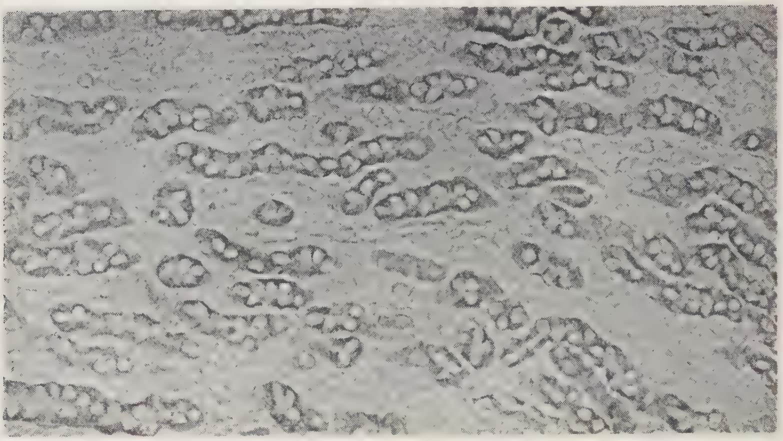

FIG. 1. Renal tubules. *In situ* indicator technic for the demonstration of succinic dehydrogenase by reduction of neotetrazolium chloride to purple formazan. ×200. Photographed by the Armed Forces Institute of Pathology, No. 54-2136.

tissue elements to provide improved histochemical tools.

Summary. A new technic for the histochemical localization of enzymes has been devised which is based on the use of an *in situ* indicator. As an illustrative example of this type of technic, succinic dehydrogenase has been demonstrated in tissue sections with neotetrazolium chloride serving as the *in situ* indicator.

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Inhibition of Melanin Formation *In vivo* by 4-Chlororesorcinol. (21437)

FREDERICK C. KULL, ROLAND BONORDEN, AND RUDOLF L. MAYER.
From the Research Department, CIBA Pharmaceutical Products, Inc., Summit, N. J.

It is well known that phenylthiourea and resorcinol derivatives display high anti-tyrosinase activity. Recently, by means of a potato-phenoloxidase spray method described by Mayer, Grimm, and Kull(1), we observed that certain resorcinol derivatives were considerably more active than resorcinol itself; the most active of these derivatives was 4-chlororesorcinol which prevented the formation of melanin from tyrosine, dopa, and epinephrine in concentrations as low as 1 x 10^{-9}(2). In view of this high antityrosinase activity, an investigation of the *in vivo* action of this and related substances as inhibitors of melanin-formation was made. Richter and Clisby(3) have shown that the prolonged ingestion of phenylthiourea eventually alters the pelt coloration in black rats. Comparative results after oral administration of p-hydroxyphenyl derivatives in guinea pigs were published by

Denton, Lerner, and Fitzpatrick(4).

Scant information is available on antityrosinase activity in coldblooded animals. Jaques (5) reported the antimelanotic action of thiouracil in regenerating tissue of axolotl tails. Although experiments in our laboratory confirmed this work, a less complex method seemed desirable. Long a favorite animal for genetical and correlated studies of normal and atypical cellular growth(6), platy fish with deep black fins were considered especially suitable for the study of antityrosinase influence on pigment. The fact that a cut dorsal fin of these fish will regenerate within 4 to 6 weeks encouraged this belief. We therefore investigated the action of 4-chlororesorcinol and other antityrosinase compounds upon the generic pigmentation of these fish as well as upon pigment formation in the developing fins.

Methods. The dorsal black fins of adult red (*Xiphophorus variatus*) and gold (*Xiphophorus maculatus*) platy fish were severed near the root. The fish were placed in non-aerated tanks of tap water containing non-toxic amounts of the test compound. Since it was ascertained by spray test assays that there was no appreciable loss of compound in the tanks during a 10 day period, the tanks were refilled weekly with new solutions. The usual diet of live and dried foods was given to all fish throughout the experiment. We observed the fish daily for fin regeneration and development of pigmentation in the newly formed dorsal fins. The estimation of the percentage of pigmentation was determined macroscopically. It represents that percentage of the developed fin showing black coloration.

Results. After one month 85% of the dorsal fin of all cut fish had been reformed, and after two months an apparently normal fin replaced the cut appendage. All regenerated fins remained unpigmented until fully formed (Fig. 1), and pigmentation of the fin-rays in control fish began only after the new fin had developed to normal size. Several weeks later the development of melanin in these same controls extended to the web-like tissue between the fin-rays; the original color was restored after 4 months.

In the presence of 4-chlororesorcinol, pigment formation was appreciably retarded in the *X. variatus* specimens and completely abolished in those of the *X. maculatus* species. Phenylthiourea also delayed pigmentation but not so markedly as 4-chlororesorcinol. Propylthiouracil was ineffective in the concentration used. As shown in Table I, the regeneration

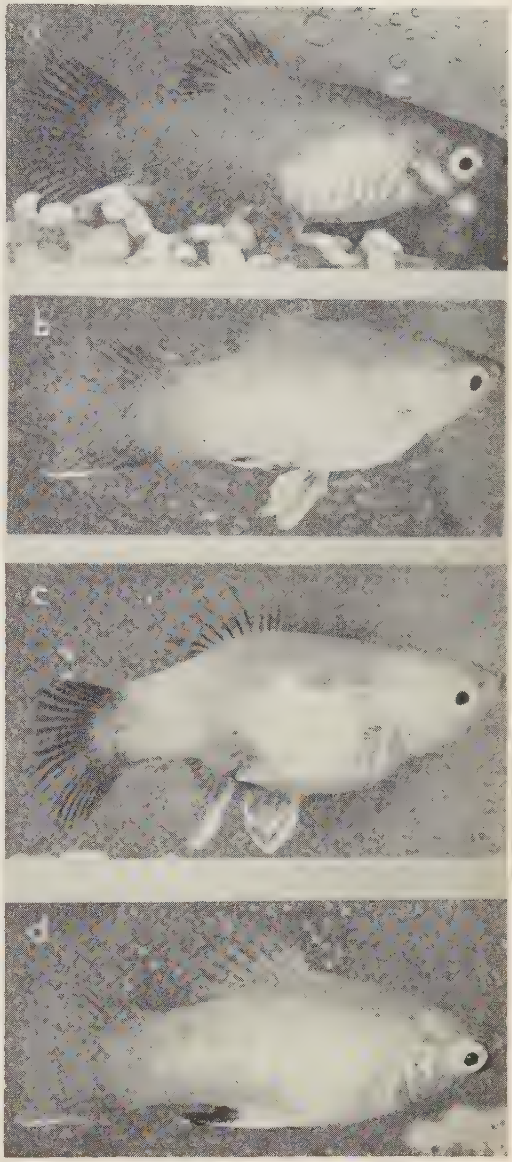

FIG. 1. Growth and pigment regeneration of dorsal fin of platy fish. a. Normal uncut control. b. After 2 wk growth with or without 4-Chlororesorcinol. c. After 6 wk growth without 4-Chlororesorcinol. d. After 6 wk growth in presence of 1.2×10^{-5} 4-Chlororesorcinol.

TABLE I. Effect of 4-Chlororesorcinol on Fin Pigmentation in *Xiphophorus maculatus* and *variatus*.

Compound	Molar conc.	No. of fish	Species	Duration of treatment 1 mo	2 mo	3 mo
				—% pigmentation—		
4-Chlororesorcinol	0 Co*	4	*X. maculatus*	0	30	60
	1.2×10^{-5}	8	”	0	0	0
	0 Co*	4	*X. variatus*	0	40	80
	1.2×10^{-5}	8	”	0	20	50
Phenylthiourea	2.2 ”	4	”	0	35	65
Propylthiouracil	2.0 ”	4	”	0	40	80

* Co = Control.

of fins was not affected by 4-chlororesorcinol, phenylthiourea, or propylthiouracil.

The fins affected by 4-chlororesorcinol remained colorless as long as the fish lived in the medicated tanks. In most of the fish, the suppression of melanin formation was reversible, and the regenerated tissue became black after the fish were replaced in unmedicated tanks. Low survival rates of the treated fish did not allow extended investigation of this phase.

In an endeavor to duplicate these results in warmblooded animals, we added 0.5% 4-chlororesorcinol to the diet of black adult or suckling C 57 mice for 60 days. This treatment had no effect on the pelt pigment. Black cats receiving one capsule (0.5%) of the substance daily for 60 days also remained unaffected.

Discussion. The observation that 4-chlororesorcinol fully arrests pigmentation in one species but only partially in another species of the same genus of fish suggests that their pigment-producing cells can convert tyrosine to melanin under different conditions. The eventual return of pigment after complete blocking of tyrosinase suggests that the *in vivo* reaction is one of competitive inhibition similar to the *in vitro* inhibition noted in manometric recordings(7). The action of 4-chlororesorcinol upon the pigment formation of fish confirm the previous finding(2) that this compound is one of the most potent inhibitors of tyrosinase.

Summary. The antityrosinase activity of 4-chlororesorcinol, first observed *in vitro*, is also present *in vivo;* this substance strongly interferes with pigment formation in the developing dorsal fin of platy fish.

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Do Soy Sterols Interfere with Absorption of Cholesterol? (21438)

H. H. Hernandez and I. L. Chaikoff.

From the Department of Physiology, University of California School of Medicine, Berkeley.

In a previous report we demonstrated that the simultaneous feeding of mixed soy sterols or of β-sitosterol with labeled cholesterol resulted in a pronounced reduction in the C^{14} recoveries in thoracic duct lymph of rats(1). We therefore stated that it is likely that plant sterols prevent hypercholesterolemia in birds fed cholesterol(2) by interfering with the absorption of the cholesterol. The conditions of our experiments should be noted. Ring labeled cholesterol was fed by stomach tube to rats into whose thoracic ducts cannulae had been inserted. Care was taken to avoid giving excessive amounts of either cholesterol or the oil in which it was administered. Thus, the amount of labeled cholesterol introduced into each rat's stomach was 4 mg dissolved in 0.25 cc of olive oil. This would be equivalent to 1.25 g of cholesterol and 70 cc of olive oil given to a man weighing 70 kilos.[†] Our interpretations were based on the C^{14} recovered in thoracic duct lymph. After the appearance of our study, Rosenman, Friedman, and Byers (3) reported experiments from which they concluded that soy sterols do not interfere with cholesterol absorption. Their experiments were carried out under conditions that differed considerably from those described above. They administered 100 mg of cholesterol in 3 cc of olive oil. Although we are anxious to avoid the impression that rat and man can be compared with regard to dietary intake, it should be pointed out that those amounts would be equivalent to 28 g of cholesterol and 840 cc of olive oil in a 70-kilo man. The conclusion of these workers was based on the amounts of digitonin-precipitable material recovered in thoracic duct lymph and giving a positive Liebermann-Burchard color reaction.

In the present report we have again studied the question of the effect of soy sterols on absorption of cholesterol. The experimental design involved small and large amounts of administered labeled cholesterol. We were thus able to measure the recoveries of both the C^{14} and the total cholesterol in thoracic duct lymph. The specificity of the digitonin-precipitable material which gives the Liebermann-Burchard color reaction is discussed.

Methods. Male rats of the Long-Evans strain, weighing 250-300 g, were used. The operative procedures employed in the present study have been described elsewhere(5). All rats were fasted for 24 hours before the insertion of the cannulae into their thoracic lymph ducts. To encourage the free flow of lymph, 0.5 or 0.9 per cent saline was provided for drinking purposes. It is shown that under these conditions the lymph flow exceeds 35 cc

TABLE I. Digitonin-Precipitable, Leibermann-Burchard Positive Materials in Lymph of Control Rats.

NaCl in drinking water, %	Lymph flow, cc	Test meal: Olive oil, cc	Test meal: Soy sterols, mg	Digitonin-precipitable, L.B. positive materials in 24 hr lymph sample (mg)
.5	38	None	None	7
”	47	”	”	8
”	35	”	”	7
”	42	”	”	8
”	40	3	”	13
”	60	”	”	11
”	51	”	”	9
”	53	”	”	21
”	43	”	”	12
”	50	”	”	26
”	52	”	”	22
”	74	”	”	38
”	63	”	”	25
.0	36	”	”	12
”	38	”	”	11
”	35	”	”	9
.9	61	”	”	22
”	57	”	”	10
”	64	”	”	12
.5	33	”	100	13
”	35	”	”	9
”	38	”	”	12
”	43	”	”	21

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[*] Aided by a grant from the Alameda County Heart Assn.

[†] The cholesterol content of the human diet varies from about 0.2 to 0.8 g depending upon the type of foods ingested(4).

for the first 24 hours (Table I). The *thoracic duct lymph* was analyzed for free and total cholesterol as follows: A 4-cc aliquot of the lymph mixture was added, dropwise, to 35 cc of a 3:1 mixture of alcohol and ether in 50-cc volumetric flasks. The mixture was maintained, with occasional shaking, at 60° for 2 hours. The volume was made to 50 cc by the addition of the 3:1 alcohol-ether solvent and then filtered through Whatman #2 filter paper. Twenty-cc aliquots were used to determine free and total sterol, as well as free and total C^{14}, according to the method previously described(5). Since radioactive compounds tend to deteriorate on standing(6), the C^{14}-cholesterol used in this study was purified before use. The method of purification was previously described(7). We are indebted to Distillation Products Industries for the mixed soy sterols which, the manufacturers report, contain 75-80% sitosterols and 10-15% stigmasterol.

Results. Amount of Liebermann-Burchard color-yielding materials in digitonide-precipitable fraction of thoracic duct lymph of control rats. Because the rats were fed various amounts of olive oil and soy sterols (and particularly since neither digitonin precipitability nor the Liebermann-Burchard (L.B.) reaction is specific for cholesterol), the importance of adequate control experiments cannot be overemphasized. Indeed, it has been shown that soy sterols are precipitated by digitonin and yield a color with the L.B. reagent(8).

Table I shows amounts of L.B. positive material in the digitonide-insoluble fraction that were present in the 24-hour lymph sample obtained from rats that received 3 cc of olive oil or 3 cc of olive oil plus 100 mg of soy sterols. From 9 to 38 mg (average, 20) of digitonin-precipitable, L.B. positive materials were found in the 24-hour lymph sample obtained from rats fed the 3 cc of olive oil. The substitution of 0.9% saline for 0.5% saline for drinking purposes appeared to have no effect on the amounts of these materials in lymph. The data in Table I suggest that the administration of the olive oil may have increased the amounts of digitonin-precipitable, L.B. positive materials in the 24-hour lymph samples. But in view of the small number of observations, no definite conclusion should be drawn on this point. We have found L.B. positive material which is also digitonin-precipitable in several commercial brands of olive oil. No appreciable effect of administered soy sterols upon digitonin-precipitable, L.B. positive materials in the 24-hour lymph sample was noted (Table I). However, β-sitosterol and stigmasterol are precipitated with digitonin, and both sterols yield a color with the L.B. reagent. We extracted 100 mg of soy sterols, exactly as described here for the lymph samples, and found them to have an apparent cholesterol value of 92 mg.

Experiment with 100 mg of administered cholesterol. Table II shows the amounts of digitonin-precipitable, L.B. positive materials recovered in the 24-hour lymph sample of six rats that had received 100 mg of cholesterol. An average of 28 mg was found in this lymph. Four of the 6 rats received the 100 mg of cholesterol admixed with cholesterol-4-C^{14} and in those animals, from 12 to 15% of the administered C^{14} (average, 13.7) was recovered in the 24-hour lymph sample.

The simultaneous administration of 100 mg of labeled cholesterol and 100 mg of soy sterols yielded values of 18 to 20 mg for digitonin-precipitable, L.B. positive materials in the 24-hour lymph samples of 3 rats (N1, N2, and N4). Although these amounts of L.B. materials might be considered significantly different from those observed in rats fed only the 100 mg of cholesterol, there can be no doubt, from the C^{14} recoveries, that the administration of 100 mg of soy sterols significantly depressed the recovery of digitonin-precipitable C^{14} in the 24-hour lymph sample.

Experiment with 4 *mg of cholesterol* again showed that while there is little difference in the amount of digitonin-precipitable, L.B. materials recovered in the 24-hour lymph samples of rats fed either 4 mg of labeled cholesterol alone or 4 mg of labeled cholesterol plus 4 mg of soy sterols, the C^{14} recoveries again leave no doubt that the administered soy sterols have a pronounced effect upon the recoveries of the C^{14} cholesterol in the lymph.

In our previous report, the percentages of the C^{14}-cholesterol in thoracic duct lymph found in the esterified form ranged from 42

TABLE II. Effect of Soy Sterols on Cholesterol Absorption in Rats.

Test meal				24-hr lymph sample			
Lymph flow, cc	Olive oil, cc	Cholesterol (mg) * 4-C^{14}-labeled † Unlabeled	Soy sterols, mg	Digitonin-precipitable, L.B. positive materials, mg	% of fed C^{14} recovered in digitonin-precipitable fraction	% of sterol esterified	
						Total	C^{14}
45	3	100†	None	27	—	42	—
64	3	”	”	29	—	56	—
34	3	100*	”	23	12.2	65	40
60	3	”	”	29	14.4	62	60
51	3	”	”	27	14.6	53	57
57	3	”	”	31	13.8	63	52
45	3	”	100	20	8.0	48	60
33	3	”	”	20	8.0	56	79
63	3	100	”	18	9.2	62	67
67	.25	4*	None	12	24.6	56	74
94	”	”	”	17	27.0	58	75
93	”	4	”	14	29.8	59	68
90	”	4*	4	10	12.4	60	61
67	”	”	”	10	13.3	51	66
88	”	”	”	20	13.4	60	54

to 63%(1). In the present study, regardless of the amount absorbed, the percentages esterified ranged from 54 to 75. The administration of the soy sterols had no effect on the percentages of the thoracic duct lymph cholesterol found in the esterified form.

Discussion. The observations reported here confirm our previous conclusion on the effects of soy sterols upon cholesterol absorption. The administration of soy sterols resulted in a significant depression of cholesterol absorption as judged by the recoveries of digitonin-precipitable C^{14} in the lymph samples collected during the first 24 hours after the administration of labeled cholesterol. This was observed in rats fed 4 mg of cholesterol as well as in rats fed 100 mg of cholesterol.

Rosenman *et al.* pointed out that their failure to confirm our finding that soy sterols inhibit cholesterol absorption might be due to their use of larger amounts of cholesterol, namely, 100 mg. It is shown here, however, that, provided conclusions are based on C^{14} studies, soy sterols are as effective at the 100-mg level as at the 4-mg level of administration. The failure of Rosenman *et al.* to find a significant difference in the amounts of digitonin-precipitable, L.B. positive material in rats fed 1) cholesterol alone and 2) cholesterol plus soy sterols is not surprising. Neither digitonin precipitation nor the L.B. color is specific for cholesterol. The L.B. test is a general color reaction for polycyclic, unsaturated compounds(8). The fact that cholesterol is not the only compound precipitated by digitonin has been repeatedly pointed out(9,10). In view of these considerations, the importance of adequate controls to take into account both the variations in the amounts of L.B. positive material normally present in lymph and the possibility of interfering substances cannot be overemphasized.

Summary. 1. The effect of soy sterols upon recoveries of digitonin-precipitable C^{14} in thoracic duct lymph of rats fed C^{14}-labeled cholesterol was studied. That administered soy sterols depress cholesterol absorption is fully confirmed, when as little as 4 mg or as much as 100 mg of cholesterol were fed. 2. The validity of absorption studies for cholesterol based on the amounts of digitonin-precipitable, Liebermann-Burchard positive materials recovered in lymph is discussed.

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Some Effects of Dietary Threonine, Tryptophan, and Choline on Liver Enzymes and Fat.* (21439)

DOROTHY ARATA, A. E. HARPER, GYDA SVENNEBY,† J. N. WILLIAMS, JR., AND C. A. ELVEHJEM.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Reports from this(1) and other laboratories (2,3) have shown that abnormal amounts of fat are deposited in the livers of rats fed a 9% casein diet containing choline and supplemented with tryptophan and methionine. When threonine was added to this diet growth was stimulated and the deposition of liver fat was reduced. The mechanism by which such an amino acid deficiency causes fat to accumulate in the liver is unknown. Similarly, little is known of the effect of an accumulation of fat on the normal metabolism of the liver. During an attempt to discover a specific biochemical defect associated with this abnormal accumulation of fat in the liver, Harper *et al.* (4) observed that the addition of threonine to a 9% casein diet caused a reduction in the deposition of fat in the liver and a concomitant increase in the activities of certain soluble liver enzymes and in endogenous oxidation and decarboxylation. A decrease in the activities of certain of the mitochondrial enzymes in the liver was also observed. The inverse relationship between the rate of endogenous respiration and soluble liver enzymes and the extent of fat deposition in the liver, and the anomalous decrease in the activities of the mitochondrial enzymes, were considered worthy of further investigation.

The present study was undertaken to explore further these preliminary observations. It was essential to establish whether the apparent relationships which had been observed between enzyme activity and fat deposition in the liver would occur under a variety of dietary conditions or whether these changes were independent reflections of the balance of amino acids in the diet. An approach to this problem was to study the effects on the activities of certain liver enzymes of several diets that were known to cause differences in liver fat deposition. The activities of certain enzyme systems in the livers of rats fed diets deficient in both choline and threonine were compared with the activities of the same enzymes in the livers of rats fed diets primarily deficient in threonine. The same enzymes were also studied in the livers of rats fed diets in which a deficiency of tryptophan was superimposed on that of threonine to give a double amino acid deficiency. It was hoped that through these dietary manipulations more information concerning the various factors influencing both the activities of the selected liver enzymes and the deposition of fat in the liver could be obtained.

Methods. Weanling male rats of the

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† Fellow of American Association of University Women.

TABLE I. Growth and Liver Fat of Rats Fed 9% Casein Diets. No. of rats in each series, 88.

Variable measured	Diets					
	Series A*		Series B†		Series C‡	
	+ threonine	− threonine	+ threonine	− threonine	+ threonine	− threonine
Growth (g/wk)	20.5 ± 1.5§	15.7 ± .6	22.5 ± 1.7	17.7 ± 1.9	18.5 ± 1.6	17.5 ± 1.3
Liver fat (% dry wt)	11.6 ± 3.0	28.3 ± 3.2	39.2 ± 3.2	39.8 ± 2.7	10.9 ± 3.4	28.1 ± 3.4

* Series A = basal ration. † Series B = basal ration minus choline. ‡ Series C = basal ration minus tryptophan. § Stand. error of mean.

Sprague-Dawley strain, weighing 40-50 g were placed in individual cages and fed the experimental rations *ad lib.* for 2 weeks. The basal diet used in these experiments consisted of the following: sucrose 81.2; casein 9.0; methionine 0.3; tryptophan 0.1; corn oil 5.0; salts IV(5) 4.0; vitamin mixture 0.25; and choline 0.15%. The vitamin mixture provided, in mg %, thiamine 0.5; riboflavin 0.5; niacin 1.0; calcium pantothenate 2.0; pyridoxine 0.25; biotin 0.01; folic acid 0.02; vitamin B_{12} 0.002; and inositol 10.0. Two drops of halibut liver oil, fortified with vitamins E and K were administered weekly to each rat. The animals were separated into 3 main groups, Series A, B, and C, each of which was divided into 2 sub-groups. In Series A, one-half of the animals were fed the basal ration, and the other half the basal ration supplemented with 0.36% DL-threonine. Series B followed the same pattern except that choline was removed from the diets of both sub-groups. Series C differed from Series A only in that tryptophan was omitted from the diet of both sub-groups. In all of these studies, 2 and sometimes 3 complete experiments were carried out. The numbers of animals used in the experiments are presented in the tables, under *Results.* At the end of the 2-week feeding period, the animals were stunned by a blow on the head and decapitated. The livers were removed as rapidly as possible, chilled in ice, and homogenized in a Potter-Elvehjem homogenizer with 5 volumes of ice cold 0.039 M sodium potassium phosphate buffer (pH 7.3). The homogenates were pipetted into the main compartment of prepared Warburg flasks kept in ice. The remaining portions of the homogenates were frozen and saved for liver fat determinations. The *enzyme systems* studied in these experiments included endogenous oxidation and decarboxylation(6), succinic oxidase(4), choline oxidase(7), tyrosine oxidase(4), and xanthine oxidase(8). Methods for the estimation of activity of these enzyme systems are given in the references listed after each enzyme. For the determination of pyruvic oxidase, 0.3 ml of 0.04 M sodium oxalacetate (prepared immediately before use; pH 7.3), 0.5 ml of 0.1 M sodium pyruvate (pH 7.3), and 0.2 ml distilled water were pipetted into the side arm of a Warburg flask. The main compartment contained 0.5 ml of Krebs-Ringer phosphate buffer(6) and 1.0 ml of the liver homogenates prepared as above. A control flask containing distilled water in place of pyruvate was run simultaneously to correct for endogenous oxygen uptake. The center well of each flask contained 0.2 ml of 10% potassium hydroxide.

Results. In all cases, the values from replicate experiments have been averaged to give the final estimation of the variables studied for each particular ration.

Growth and Liver Fat. Average weekly gain and average per cent of liver fat for the various groups are presented in Table I. The addition of 0.36% DL-threonine to the basal diet (Series A) increased the growth rate and markedly reduced liver fat deposition. In Series B, when choline was removed from the basal ration, the addition of threonine caused a growth stimulation similar to that observed in Series A but it did not prevent the accumulation of fat in the livers. When supplementary tryptophan was omitted from the basal diet (Series C), no growth stimulation from additional threonine occurred; but the threonine caused a reduction in the fat content of the liver to a normal level. Hence, by manipulating the dietary content of threonine, choline, and tryptophan, it was possible to

TABLE II. Effect of Adding Threonine to Basal Ration on Activity of Several Enzymes of the Liver. Series A (basal).

Enzyme	Total No. animals	Oxygen uptake	
		+ threonine	− threonine
		μl O_2/hr/g fresh tissue	
Endogenous oxidation	24	831 ± 159*	491 ± 100
” decarboxylation	24	650 ± 161	405 ± 51
Succinic oxidase	22	3409 ± 177	3935 ± 164
Choline ”	22	1668 ± 87	1795 ± 113
Pyruvic ”	22	1108 ± 84	1348 ± 155

* Stand. error of mean.

control both growth and liver fat deposition and thus to study the relationship of each of these variables to the enzyme systems of the livers.

Activity of Liver Enzyme Systems. The results of the enzyme studies are summarized in Tables II, III, IV. When both choline and tryptophan were present in the diet (Table II), the inclusion of 0.36% DL-threonine caused a significant increase in endogenous oxidation and decarboxylation. In these experiments there was no consistent effect of threonine on the activities of the mitochondrial enzymes, succinic oxidase, choline oxidase, and pyruvic oxidase. In some cases, these enzymes were more active when threonine was fed, and in some cases they were less active, when compared with the same enzymes in rats fed a threonine deficient ration. These findings are not consistent with those of a previous study(4) in which the activities of the succinic and choline oxidases were found to be somewhat reduced when threonine was included in the ration. In view of the difference between the results of the two studies on the mitochondrial enzymes, an attempt was made to find an explanation for the inconsistency. It was noted that, in general, an inverse relationship between activity of the mitochondrial enzymes and endogenous oxidation existed, and, when succinic oxidase activity was plotted against endogenous oxygen uptake the curve presented in Fig. 1 was obtained. The same relationship between endogenous oxidation and enzyme activity was found to hold both for choline and pyruvic oxidases. Thus, the inconsistency between the results of the present studies and those reported previously for succinic and choline oxidases(4) may be attributed to the effects of endogenous oxidation on the measurement of these enzymes. When the absolute endogenous oxidation was very high, as it was in some of the earlier experiments(4), it is possible that the hydrogen-transport systems which are required for the oxidation of endogenous substrates were too limited to permit maximal activities of the specific oxidative enzymes under study in addition to the endogenous oxidation. In the experiments reported in this paper, in which the absolute values for the endogenous oxidation were generally low, the succinic and choline oxidase activities would be expected to approach the maxima. It was not considered necessary to repeat the determination of xanthine oxidase and tyrosine oxidase

TABLE III. Effect of Adding Threonine to Basal Ration Minus Choline on Enzyme Activity of the Liver. Series B (basal-choline).

Enzyme	Total No. animals	Oxygen uptake	
		+ threonine	− threonine
		μl O_2/hr/g fresh tissue	
Endogenous oxidation	24	720 ± 157*	449 ± 83
” decarboxylation	24	517 ± 140	305 ± 76
Tyrosine oxidase	12	321 ± 31	167 ± 43
Xanthine ”	12	71 ± 7	39 ± 5
Succinic ”	24	3396 ± 274	3699 ± 217
Choline ”	24	1537 ± 111	1420 ± 154
Pyruvic ”	12	1434 ± 134	1229 ± 41

* Stand. error of mean.

TABLE IV. Effect of Adding Threonine to Basal Ration Minus Tryptophan on Enzyme Activity of the Liver. Series C (basal-tryptophan).

Enzyme	Total No. animals	Oxygen uptake	
		+ threonine	− threonine
		μl O_2/hr/g fresh tissue	
Endogenous oxidation	40	954 ± 150*	849 ± 160
" decarboxylation	40	737 ± 140	752 ± 195
Xanthine oxidase	16	90 ± 6	86 ± 5
Tyrosine "	16	355 ± 31	373 ± 58
Succinic "	22	3194 ± 229	3041 ± 253
Choline "	22	1762 ± 253	1670 ± 192
Pyruvic "	16	1474 ± 212	1085 ± 196

* Stand. error of mean.

activities in Series A because the values for these 2 enzyme systems in Series B compared favorably to those published previously(4) and because the hydrogen-transport systems for these enzyme systems are different from those utilized for the main part of endogenous respiration. Thus xanthine oxidase and tyrosine oxidase should not be affected by changes in the absolute rate of endogenous respiration.

When choline was omitted from the basal ration (Series B), the addition of threonine significantly increased the endogenous oxidation and decarboxylation and the activities of the 2 soluble enzyme systems, tyrosine oxidase, and xanthine oxidase (Table III) but did not lower the fat content of the liver (Table I). Thus the changes in these enzyme systems were similar to those which resulted from threonine supplementation in Series A. Endogenous respiration and the 2 soluble enzymes were stimulated by the addition of threonine to the diet in spite of the fact that the livers from both the supplemented and the basal groups contained similar amounts of fat. It appears then, that the accumulation of fat in the liver does not, in itself, cause a depression of the endogenous respiration and the 2 soluble enzyme systems studied.

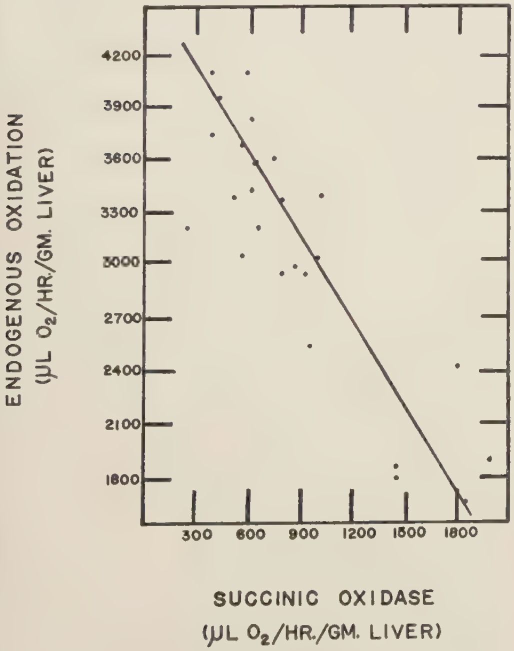

FIG. 1. Relationship between activity of succinic oxidase and rate of endogenous oxidation.

Although the presence or absence of choline had no effect on the enzyme systems studied (compare Series A and B), the tryptophan content of the ration appeared to exert a major influence on the activities of the 4 systems shown to be reduced as a result of a dietary deficiency of threonine. In the absence of tryptophan, endogenous oxidation and decarboxylation, xanthine oxidase, and tyrosine oxidase were as active in the unsupplemented group as in the threonine supplemented group (Table IV). However, the addition of threonine to the ration still markedly depressed the fat content of the liver.

Discussion. Since there was no consistent correlation between the activities of the enzymes studied and the extent of fat deposition in the liver, the differences that were observed are apparently unrelated reflections of the nutritional conditions imposed on the animals. Of particular interest are the effects of the amino acid deficiencies.

Tryptophan and threonine are apparently equally limiting for growth in 9% casein diets supplemented with methionine, since growth was increased only when one of these amino acids was added to the diet in the presence of the other. When tryptophan was present, threonine not only became limiting for growth but also became limiting for the control of fat deposition and for the activity of certain enzyme systems (*viz.*, xanthine and tyrosine oxidases and endogenous respiration). When threonine was added to the diet containing tryptophan, the rate of endogenous respiration and the activity of xanthine oxidase and tyrosine oxidase increased while the fat content of the liver decreased. Thus, in agreement with the previously published report(4), an inverse relationship was apparent between the activities of these enzymes and liver fat deposition.

When a dietary deficiency of choline was superimposed upon the threonine deficiency, however, this relationship between enzyme activity and fat deposition disappeared. In this case, although the rates of endogenous oxidation and decarboxylation and the activities of the xanthine oxidase and tyrosine oxidase systems increased when threonine was included in the diet, liver fat deposition was high in both groups. Since the mechanism by which a choline deficiency causes fat to accumulate in the liver probably differs from that of an amino acid deficiency these results are not surprising. They do, however, show that the accumulation of fat does not, in itself, affect the activities of the enzymes studied; and conversely, that the changes observed in the enzyme systems are not necessarily accompanied by a concomitant change in liver fat deposition.

On the other hand, the results obtained when tryptophan was omitted from the diet were not anticipated. Since, as had been observed previously, the fat content of the liver was reduced when threonine was added to the tryptophan-deficient diet, tryptophan apparently did not become limiting for the control of fat deposition even though it became limiting for growth. It is interesting to observe that endogenous oxidation and decarboxylation and the soluble cytoplasmic enzymes of the liver are controlled strongly by threonine when supplementary tryptophan is given but not when tryptophan is omitted. In fact, the enzyme systems showed normal activity in both of the tryptophan-deficient groups. Thus it appears that the enzyme changes observed are more directly linked to a deficiency of threonine rather than to the abnormal metabolism of fat resulting from a partial deficiency of that amino acid. In Series C, the limited supply of tryptophan would undoubtedly reduce the rate of synthesis of enzymes, hormones, and other protein molecules for which tryptophan is an essential constituent. This might release enough threonine for the normal synthesis of the enzyme systems studied in these experiments, which are apparently more dependent on threonine than on tryptophan.

Although the enzyme changes observed in these experiments could not be directly related to the accumulation of fat in the liver, it is quite possible that other enzyme systems, which have not yet been studied, are involved in this condition. It is apparent from these results, however, that the balance of amino acids required to maintain one bodily function in a normal state differs quite markedly from that required for another.

Summary. 1. Several liver enzyme systems were studied in rats fed diets known to produce fatty livers in order to determine whether changes in liver fat deposition could be associated with specific enzyme changes. The activities of the soluble cytoplasmic enzymes, xanthine oxidase, and tyrosine oxidase, as well as the rate of endogenous oxidation and decarboxylation, were found to be markedly increased when supplemental threonine was included in a 9% casein ration containing added methionine and tryptophan with or without choline. Several mitochondrial enzyme systems, choline oxidase, succinic oxidase, and pyruvic oxidase showed a sufficiently great variation from experiment to experiment to prevent any differences in the activities of these enzymes from being considered significant. This variation has been attributed to an inverse relationship between the activities of these enzymes and the absolute rate of endogenous oxidation in the liver. 2. When supplemental tryptophan was removed from the ration, all of the differences in enzyme activity between threonine-deficient and threonine-

supplemented animals disappeared completely, although the changes in liver fat deposition produced by adding or omitting threonine were unaltered under these conditions. It is suggested that the balance of amino acids in the diet is the primary factor responsible for the enzyme changes observed and that the accumulation of fat in the livers bears no direct relationship to those enzyme changes.

The authors would like to express their gratitude to William Monson, Duane Benton, and Marie Winje for their valuable assistance in various phases of this work.

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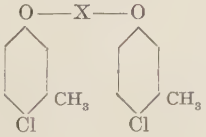
Ascaricidal Action of Cadmium. (21440)

CARL A. BUNDE, HARRY E. BLAIR, GEORGE R. BURCH, AND JOHN W. LEE.
From the Research Department, Pitman-Moore Co., Indianapolis, Ind.

In the process of screening 2 series of compounds of the general structure



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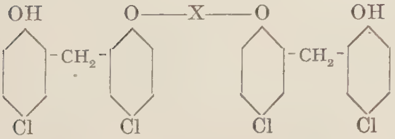

where *X* was a bivalent heavy metal, ascaricidal activity was noted in those compounds where X = Cd. The preliminary *in vitro* data on swine ascarids and *in vivo* data on canine ascarids suggested a relationship of antihelminthic activity to Cd content of the respective compounds. Pursuing this concept further, a number of Cd compounds were evaluated for activity against ascarids in pigs by the addition of the compounds to dry ground feed.

Method and results. The desired concentration of Cd in the feed to be eaten was obtained by pre-mixing CdO in 1% of the final volume desired and then carefully diluting this concentrate to 100 times its volume with whatever feed the test animals were eating as a regular ration. Various concentrations were used initially, but it soon became evident that a final concentration of more than 0.03% was unpalatable to some animals and concentrations as high as 1% caused emesis and refusal to eat. Since it was necessary to have a reasonably predictable intake of Cd, concentrations of less than 0.03% were employed for

TABLE I. Effect of Cadmium in Feed on Amount of Food Eaten.

		Food eaten/24 hr/pig	
No. of pigs	Avg wt, lb	Unmedicated, lb*	Medicated, lb*
8	54	2.25	2.31 (.02% CdO, 2 days)
6	56	2.50	2.66 " " 3 "
6	49	1.84	2.60 .015% " 3 "
8	42	2.65	2.30 " " 3 "

* Unmedicated based on 1 day's feed. Medicated based on dosing period (2 or 3 days).

TABLE II. Measure of Ascarid Elimination by Addition of CdO to Feed.

No. of pigs	CdO in feed	Worms passed and found	Worms found at autopsy	% effective compared to Controls	
				Controls	Worms passed
8*	0	0	159		
14	.015%, 3 days	59	1	99	98
13	.02%, 3 "	59	9	96	87
8	.02%, 2 "	49	23	86	68

* All from same herd. Fecals from this herd were all strongly positive for ascarids.

efficacy studies. Table I shows that concentrations of .015% and .02% CdO in feed is eaten as readily as unmedicated feed.

Efficacy studies were of 2 types. In the first method, treatment of pigs known to have ascarids were followed by autopsy and the number of worms found in the gut was compared to autopsied untreated controls from the same herd. Table II presents data obtained in this manner. In the other measure of efficacy, herds known to harbor ascarids from fecal floatation tests(1) made on stools were treated and the worms passed were collected and counted. Three herds totaling 238 pigs were treated and a total of 1710 ascarids was passed and recovered. Treatment consisted of .01% to .02% CdO in the feed for 3 days. Each herd acted as its own control in that no worms were noted in the yards and pens during the week preceding treatment and all worms collected were passed during the week following treatment.

Discussion. Cadmium is a strong emetic (2,3) so its use as an antihelminthic by capsule or tablet administration might be very limited. However, as an additive to feed in minute amounts, the emetic concentration in the stomach is not reached. As seen here, such concentrations effectively eliminate ascarids from pigs when the animals are given no other feed for a 2 or 3 day period.

Conclusions. CdO mixed with dry ground grain and supplied as the only food for two or three days will effectively eliminate ascarids from pigs. The concentration shown to be effective is .01% to .02% and this mixture is palatable and readily eaten.

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Presence of Glutamine and Asparagine in Enzymatic Hydrolysates of Oxytocin and Vasopressin. (21441)

H. CLAIRE LAWLER, STERLING P. TAYLOR,* AILSA M. SWAN, AND VINCENT DU VIGNEAUD.†

From the Department of Biochemistry, Cornell University Medical College, New York City.

The structure of oxytocin proposed by du Vigneaud, Ressler and Trippett(1) was based on a body of experimental evidence obtained in this laboratory which showed clearly the arrangement of the 8 amino acids in the form of a cyclic pentapeptide containing cystine, one-half of the cystine moiety possessing a free amino group and the other half being linked through its amino group to the rest of the cyclic portion of the molecule and through its carboxyl group to the tripeptide prolylleucylglycine. Evidence was also derived that the terminal glycine carried an amide grouping, and it was assumed that the other 2 moles of ammonia present in the hydrolysate of oxytocin had arisen from amide linkages formed with the carboxyl groups of glutamic acid and aspartic acid. It was further assumed that glutamine and asparagine moieties were present rather than their isomers. These assumptions were likewise embodied in the proposal for the structure of vasopressin by du Vigneaud, Lawler and Popenoe(2). The same assumptions regarding glutamine and asparagine were also made in the structures independently proposed by Tuppy(3) for oxytocin and by Acher and Chauvet for vasopressin(4). The octapeptide amide structure proposed for oxytocin was synthesized and the extensive comparisons of the physical, chemical and biological properties of the synthetic product with those of the purified natural oxytocin led to the conclusion that the synthetic product was oxytocin(5,6). However, it was felt that it would still be of interest to obtain degradative evidence for the existence of glutaminyl and asparaginyl residues in the natural product.

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* Deceased.

† The authors wish to express their appreciation to Lederle Laboratories Division, American Cyanamid Co., for research grants which have aided greatly in this work.

The broad spectrum of proteolytic activity associated with papain(7) suggested its use for these degradation studies. Furthermore, papain had been shown to hydrolyze the α amide linkage but not to affect the γ amide linkage of carbobenzoxy-L-glutamic acid diamide(8). The crystalline papain prepared by Kimmel and Smith(9) was found by them to have a specificity which resembled that of the cruder preparations. Oxytocin and vasopressin were therefore digested with crystalline papain.‡ Evidence was obtained for the presence of asparagine and glutamine in both papain hydrolysates.

Experimental. Highly purified preparations of oxytocin and vasopressin were incubated with the enzyme. To 10 mg of oxytocin dissolved in 6.8 ml of acetate buffer, pH 5.3, were added 0.6 ml of 0.0074 M Versene at pH 4.6. Hydrogen sulfide was passed into the mixture and then a solution of crystalline papain, 0.73 mg of protein N in 0.6 ml, was added. The mixture was treated with hydrogen sulfide and the tube was sealed. At the end of 18 hours at 38° the tube was opened and the reaction mixture aerated. The hydrolysate was assayed according to the United States Pharmacopeia method(10) and was found to be inactive. A portion of the hydrolysate was applied to Whatman No. 4 chromatographic paper for two dismensional chromatography in 5:1:5 *n*-butyl alcohol-acetic acid-water and 75% phenol. Similar chromatograms were prepared from various mixtures of glycine with glutamine, asparagine, isoglutamine and isoasparagine. The chromatograms were developed with ninhydrin. Included in the spots which appeared on the chromatogram of the papain hydrolysate were two which corresponded in position to those of the authentic glutamine and asparagine in relation to the glycine spot. The spot from the oxytocin hydrolysate in the asparagine position had a gray color which we obtained under these conditions with asparagine in contrast to the purplish color when isoasparagine was used in the mixture of amino acids.

Another portion of the enzymatic hydrolysate which was equivalent to 4.6 μM of hydrolyzed oxytocin was dried and chromatographed on starch(11) using the eluting solvent 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 *N* lithium chloride.§ Alternate tubes were analyzed and peaks were found that corresponded in position to the peaks produced by asparagine and glutamine. When prepared mixtures of asparagine, glutamine, and their isomers along with glycine had been chromatographed in this solvent, resolution with good recoveries had been obtained. The amounts of asparagine and glutamine as calculated from the areas under the peaks(12) in the chromatogram of the papain hydrolysate represented 30 and 20%, respectively, of that theoretically derivable from the oxytocin used.

Since the positions of the peaks of glutamine and asparagine fell close to those of their isomers in the starch chromatogram, identification of the peaks when only two were present was difficult. Therefore, further identification of the chromatographed material by zone electrophoresis on filter paper was undertaken. The remainder of the material in the peaks from the starch column chromatogram, representing what was believed to be the glutamine and asparagine, was therefore combined and dried. The material in the peaks formed by the synthetic amino acid mixture was treated in a similar fashion. The dried material was taken up in water, applied to Whatman No. 4 chomatographic paper strips and chromatographed in 75% phenol in order to remove the lithium chloride of the eluting solvent. The material in the entire area corresponding to the amino acids was eluted with water, dried, and subjected to zone electrophoresis on filter paper. The material was applied in a small volume to Whatman No. 3 MM filter paper saturated with sodium bicarbonate buffer, pH 8.3, ionic strength 0.1. A closed system was used to prevent evaporation. Four hundred volts were applied for 1.5 hours over a distance of 42 cm at room temperature. The paper strips were developed with a ninhydrin solution which contained collidine. Under these conditions glutamine and asparagine travel at a slower rate than their corresponding isomers. The material in the peaks from the starch column eluate obtained from the oxytocin hydrolysate behaved identically with the authentic samples of asparagine and glutamine.

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‡ The sample of crystalline papain used was made available to us by Professor Emil L. Smith to whom we wish to express our appreciation.

§ This solvent mixture was suggested by Professors Moore and Stein.

A sample of vasopressin was subjected to hydrolysis by papain under conditions similar to the ones described for the hydrolysis of oxytocin except that less enzyme was used (equivalent to 0.11 mg protein N). A fraction of the hydrolysate was chromatographed on paper and the developed chromatogram had spots which corresponded to the spots produced by asparagine and glutamine. The hydrolysate on starch column chromatography was found to produce peaks which corresponded in position to the peaks produced by these substances. The yields from vasopressin were calculated to be approximately 20% for glutamine and for asparagine.

Summary. In papain hydrolysates of oxytocin and vasopressin, asparagine and glutamine were found to be present. The asparagine and glutamine were identified by chromatography on paper and starch.

The authors wish to thank Mr. Richard Murphy for his assistance in the starch column chromatography.

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Antipneumococcal Cross Reactions of Antigens of *Leuconostoc mesenteroides.*[*] (21442)

JAMES M. NEILL AND ALEXANDER H. PINKES.

From the Department of Bacteriology and Immunology, Cornell University Medical College, New York City.

Previous papers(1-4) have described the antipneumococcal cross-reactions of dextrans of a large number of strains of *Leuconostoc mesenteroides*. The present paper deals with antipneumococcal cross-reactions of other antigens of *Leuconostoc mesenteroides*. These antigens are not dependent upon sucrose for their formation and hence are more comparable to antigenic components of other bacteria than dextrans are. For convenience, the term "non-dextran" antigens is used to denote these components of the leuconostoc bacteria, although that term has the disadvantage of not excluding the levans(5) which some strains may produce, in addition to dextran, when grown in sucrose-containing mediums. Non-dextran antigens have been studied in respect to their reactions with leuconostoc antiserums, by Hucker(6) whose results revealed serological diversity of various strains, and recently by McCleskey and associates(7) who indicated strain diversity by setting up various serological "types." Since antipneumococcal reactions had proved useful in characterization of dextrans(1-4), we made a preliminary survey on pneumococcal relationships of non-dextran antigens of a small collection of strains, from which two were selected for the present investigation. The strains are NURB B512-F which cross reacts reciprocally with type 27 pneumococcus, and No. 6[†] which cross reacts reciprocally with type 32 pneumococcus. The B512-F is commonly employed in commercial production of dextran plasma volume extenders. The No. 6 strain is the one used in original demonstrations of antipneumococcal reactions of dextrans(1) and of enzymatic synthesis of dextrans(8).

Materials and methods. The non-dextran leuconostoc antigens which consisted of suspensions of the bacterial cells and of HCl extracts prepared from the cells, were obtained from glucose broth cultures in which no dextran had been formed. The suspensions used for the agglutination tests and for the production of the leuconostoc antiserums were heated at 65-70°C for 30 minutes. The HCl extracts were prepared from 1.0% suspensions of dried bacterial cells in salt solution containing 0.05N HCl, which were heated 30 minutes at 55°C, boiled for 10 minutes, and then centrifuged several times after icebox storage until stable clear solutions were obtained. Dr. H. M. Tsuchiya of the Northern Utilization Research Branch supplied a large amount of the bacterial cells of both the leuconostoc strains. The dextran of the B512-F strain was furnished by Dr. Carl Rist and associates at the Northern Utilization Research Branch; the dextran of the No. 6 strain, by Dr. Edward J. Hehre of this department. All the antiserums were from rabbits. Most of the antipneumococcal serums were supplied by Dr. H. D. Piersma, Dr. J. K. Miller, the late Dr. H. W. Lyall, and Dr. G. I. Steffen. The agglutination test mixtures were shaken in a Kahn machine for 30 minutes at 20-22°C and then stored in the icebox for 3 to 4 days when the final observations were made. The precipitation test mixtures were incubated 2 hours at 37°C (without mechanical shaking) and stored in the icebox for 4 to 5 days before the final observations.

Results. Pneumococcal antiserums vs. leuconostoc antigens. Agglutination and precipi-

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[†] This strain has been called B in previous papers from this department. It is called No. 6 (its original designation in our culture collection) in the present paper in order to avoid confusion with the name of the B512-F strain. The No. 6 strain was isolated by us in 1939 from an over-ripe banana. The B512-F strain was supplied by Northern Utilization Research Branch of the Department of Agriculture, where it had been isolated from a sample of spoiled root beer.

TABLE I. Results* of Tests of Pneumococcal Antiserums versus Non-Dextran Antigens and Dextrans of *L. mesenteroides* Strains B512-F and No. 6.

	Non-dextran antigens				Dextrans	
	Agglutination of bact. cells		Precipitation of HCl extracts		Precipitation of solutions	
Pneumococcal antiserums	B512	No. 6	B512	No. 6	B512	No. 6
Type 27	640	<5	20	0	<5	<5
4	10	<5	U	0	<5	<5
16	10	<5	U	0	<5	<5
32	0	1280	0	40	<5	<5
23	0	40	0	5	<5	<5
31	0	20	0	3	<5	<5
22	0	10	0	U	10	10
2	0	<5	0	0	160	160
20	0	<5	0	0	160	160
12	0	<5	0	0	<5†	<5†
Other types	0	<5‡	0	0	<5	<5
Rough	0	<5	0	0	0–5	0–5

* Results expressed as reciprocal of highest dilution of serum that gave positive results when mixed with equal volume of test antigen. U = undiluted serum; 0 = negative with undiluted or diluted serum; <5 = negative with 1:5 and higher dilutions of serum (positive reactions with lower dilutions were not significant because undiluted normal rabbit serums frequently agglutinate the No. 6 strain or precipitate dextrans).

† Type 12 antiserums precipitate dextrans of some strains of leuconostoc bacteria (1–4).

‡ Type 14 antiserums agglutinate Strain No. 6 with 1:5 or 1:10 dilutions of either unconcentrated or concentrated serum samples.

tation tests with leuconostoc antigens were made against a comprehensive collection of antipneumococcal serums; each type from 1 to 32, except 21 and 26, was represented by 3 to 7 samples; both concentrated and unconcentrated serums were included. The general pattern of the results is shown in Table I by the data obtained with a series of concentrated antiserums, each of which was a pool from a large number of rabbits. It is evident that the type 27 antiserum was highly reactive and the types 4 and 16 antiserums were weakly reactive against the non-dextran antigens of the B512-F strain; whereas, against the corresponding antigens of the No. 6 strain, the type 32 antiserum was highly reactive and the types 23, 31, and 22 antiserums were moderately to weakly reactive. Since types 27 and 32 were the only ones that gave positive reactions with all samples of unconcentrated antiserums, the relationship to those types can be regarded as the dominant feature in the pneumococcal cross-reacting pattern of the non-dextran antigens of the B512-F and of the No. 6 strain, respectively. In contrast, the dextrans of both strains were characterized by strong cross reactions with types 2 and 20 antiserums; and, with the exception of some reactivity with type 22, gave no significant reactions with any of the types of pneumococcal antiserum that cross-reacted with the non-dextran antigens of either of the leuconostoc strains. This distinction emphasizes the separability of the serological properties of the dextrans from those of the other antigens of leuconostoc bacteria; and, in addition, illustrates the sharp serological differences that may occur between non-dextran antigens of leuconostoc strains which synthesize serologically similar‡ dextrans. This latter point is analogous to the difference in serological properties, reported by Horsfall(9), that occurred between the capsular polysaccharides of two strains (types) of *S. salivarius* which synthesize serologically similar levans.

Anaphylaxis. That dextrans will produce anaphylaxis in guinea pigs sensitized with type 2 pneumococcal antiserum was demonstrated by Hehre and Sugg(10). The results of the

TABLE II. Anaphylaxis to Intravenous Injections of Non-Dextran Antigens or of Purified Dextrans.*

	Non-dextran antigens†		Dextrans	
Antiserum	B512-F strain	No. 6 strain	B512-F strain	No. 6 strain
Type 27	+	0	0	0
32	0	+	0	0
2	0	0	+	+
None	0	0	0	0

+ = All guinea pigs tested died within 5 min. with signs typical of acute anaphylaxis; 0 = all guinea pigs tested gave entirely negative reactions.

* Sensitization was by intraper. inj. of 0.5 ml of antiserum; challenge was by intrav. inj. of 0.5 ml of 1:10 dilution of the B512 HCl extract, 0.5 ml of undiluted No. 6 HCl extract, or 1.0 ml of 1:5,000 dilution of dextrans.

† HCl extracts prepared from bacteria grown in glucose broth (described under "Methods").

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‡ Although similar in respect to antipneumococcal cross reactions, the dextrans of these two strain do differ from each other in degree of reactivity with *S. typhi* and some other *Salmonella* antiserums, particularly in complement fixation tests(3).

TABLE III. Results* of Tests of Leuconostoc Antiserums versus Pneumococcal Antigens.

Serums† of rabbits immunized with	Agglutination Type 27 cells	Agglutination Type 32 cells	Precipitation Type 27 S	Precipitation Type 32 S
Strain B512-F	20–160	0	5–20	0
No. 6	0	20–80	0	5–10

* Results expressed as reciprocal of highest dilution of antiserum that gave positive results; 0 = negative with undiluted or diluted serum.

† 5 rabbits were immunized with each strain; the pre-immunization serums gave negative results against all of the antigens.

experiments given in Table II show that the non-dextran antigens of the B512-F and No. 6 leuconostoc strains also can produce anaphylaxis in appropriately sensitized guinea pigs. The distinctions between the non-dextran antigens and the dextrans in respect to the type of pneumococcal antiserum that sufficed for the sensitization are the same as in the precipitation and agglutination reactions of Table I.

Leuconostoc antiserums vs. pneumococcal antigens. Antiserums produced by immunization with the B512-F and the No. 6 leuconostoc strains were tested for capacity to agglutinate types 27 and 32 pneumococci and to precipitate purified types 27 and 32 S substances. As shown in Table III, the B512-F antiserums were reactive with the type 27 antigens but non-reactive with the type 32 antigens; whereas, the No. 6 antiserums were reactive against the type 32 antigens but non-reactive with the type 27 antigens. These reactions are analogous to the reactions of the types 27 and 32 antiserums with the leuconostoc antigens (Table I), and establish the reciprocal nature of the relationship of the B512-F and No. 6 strains to types 27 and 32 pneumococci.

Absorption, inhibition, and Quellung experiments. The previous data strongly indicate that the pneumococcal part of the reciprocal cross reactions is due to type-specific antibodies and antigens. Evidence that non-type-specific pneumococcal antibodies and antigens do not participate was obtained by absorption experiments, in which repeated treatments with Rough and with various other types (2, 20,16) of pneumococcal cells had no appreciable influence upon the capacities of types 27 and 32 antiserums to cross react with the leuconostoc antigens nor upon the capacities of the leuconostoc antiserums to cross react with types 27 and 32 antigens. More direct evidence was obtained by inhibition experiments.§ As shown by the summary in Table IV, the type 27-B512-F cross-agglutination reactions were inhibited specifically by the type 27 S; whereas, the type 32-No. 6 cross agglutinations were inhibited specifically by the type 32 S. The S substances of other types (2,20,14), the pneumococcal C and Cx substances, and the dextrans of the two leuconostoc strains showed no inhibitory action. The selectivity of the inhibition by the S substances, when combined with the data of all the preceding experiments, constitute good proof that the type specific anti-S is the antibody responsible for the cross-reactions of types 27 and 32 pneumococcal antiserums with the leuconostoc bacteria; and, conversely, that the type-specific capsular

TABLE IV. Specific Inhibition of the Cross-Agglutination Reactions.

Cross-reacting serums and bacterial suspensions	Substances tested* for inhibitory action Type 27 S	Type 32 S	Others†
Type 27 serum vs. B512-F bact.	+++	0	0
B512-F serum vs. type 27 bact.	+++	0	0
Type 32 serum vs. No. 6 bact.	0	+++	0
No. 6 serum vs. type 32 bact.	0	+++	0

* 1:1,000, 1:10,000, and 1:100,000 dilutions of each substance were added to separate tubes containing a series of dilutions of antiserum, prior to agglutination test.

† Types 2 S, 20 S, 14 S, pneumococcal C and Cx, and dextrans of B512-F and No. 6 strains.

+++ = Agglutination titer reduced at least 5 two-fold serum dilutions by 1:1,000, and at least 2 two-fold serum dilutions by 1:10,000 dilutions of the S substances. 0 = No reduction in agglutination titer by 1:1,000 or higher dilutions of the substances tested.

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§ S substances in these experiments were supplied by Dr. Rachel Brown of the New York State Department of Health; the pneumococcal C and Cx (11,12) were supplied by Dr. Walter F. Goebel and Dr. Maclyn McCarty of the Rockefeller Institute. The types 27 and 32 S were proved not to contain significant amounts of C or similar non-type-specific substances by tests against Rough pneumococcal antiserum.

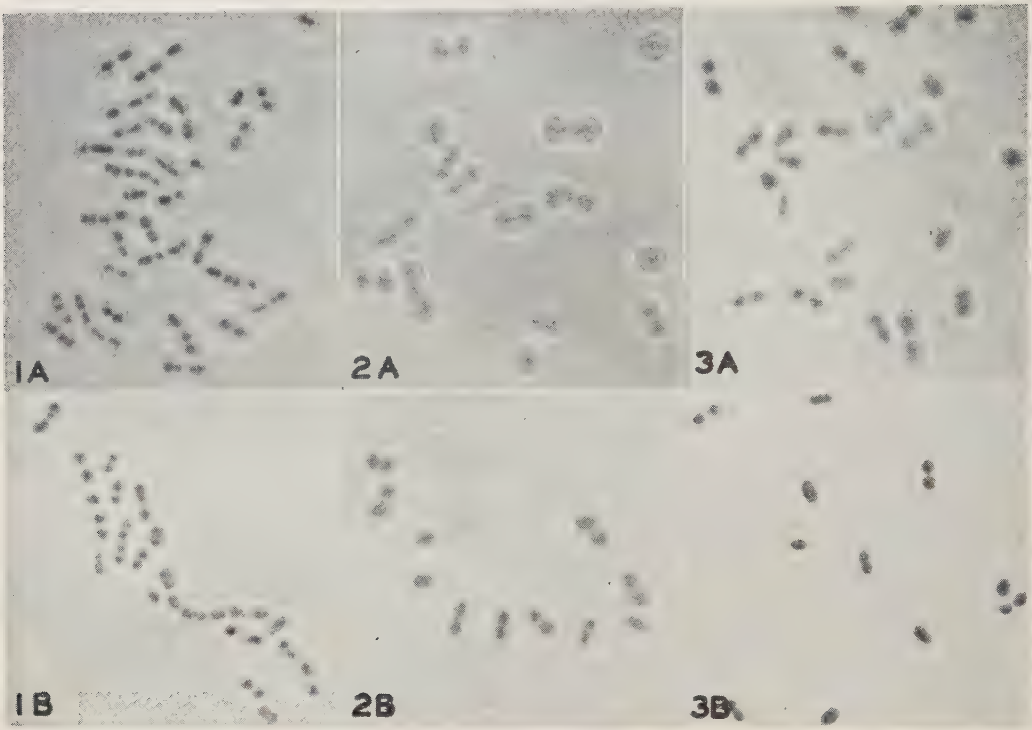

FIG. 1A. Cells from glucose broth culture of B512-F strain of *L. mesenteroides* plus type 27 pneumococcal antiserum. FIG. 1B. Cells from same leuconostoc culture plus type 32 pneumococcal antiserum.

FIG. 2A. Cells from infusion broth culture of type 27 pneumococcus plus antiserum of B512-F strain of *L. mesenteroides.* FIG. 2B. Cells from same pneumococcal culture plus antiserum of No. 6 strain of *L. mesenteroides.*

FIG. 3A. Cells from infusion broth culture of type 32 pneumococcus plus antiserum of No. 6 strain of *L. mesenteroides.* FIG. 3B. Cells from same pneumococcal culture plus antiserum of B512-F strain of *L. mesenteroides.*

Magnification in all figures is ×2160. All cells stained with safranin.

polysaccharide(S) is the antigen involved in the cross reactions of type 27 and 32 pneumococcal cells with the leuconostoc antiserums. In *Quellung tests,* strongly positive reactions were obtained with the B512-F leuconostoc bacteria against type 27 antiserums (Fig. 1), with type 27 pneumococcal cells against B512-F leuconostoc antiserum (Fig. 2) and also with type 32 pneumococcal cells against No. 6 leuconostoc antiserums (Fig. 3). The No. 6 leuconostoc bacterial cells, however, failed to give definitely positive Quellung reactions both in tests against type 32 antiserum and also in tests against the homologous antiserum, although both these antiserums had strong capacities to agglutinate suspensions and to precipitate soluble antigens of the No. 6 strain. The Quellung of the B512-F strain proves that the type 27-reactive antigens of these leuconostoc bacteria are capsular constituents occupying a place in the leuconostoc cell comparable to the place of the type specific S substance in the usual encapsulated pneumococcal cell. Apparently, the No. 6 leuconostoc do not have a sufficient amount of the type 32-reactive antigens on their cellular surfaces to give detectable Quellung reactions, although it is evident that they do possess enough of those antigens to produce antiserums which give good Quellung reactions with encapsulated type 32 pneumococci (Fig. 3) and which precipitate solutions of the type 32 S substance (Table III). These relationships on the part of the No. 6 leuconostoc strain are analogous, in principle, to the relationships observed by MacLeod and Krauss(13) with

TABLE V. Serological Tests for Non-Dextran Antigens and Dextrans Contained in Supernatant Fluids from Glucose and Sucrose Broth Cultures of Leuconostoc Bacteria.

Pneumococcal antiserum	Highest dilution of culture fluid that gave precipitation			
	Glucose		Sucrose	
	B512-F	No. 6	B512-F	No. 6
Type 27	100	0	30	<10*
32	0	3	0	<10*
2	0	0	20000	20000

* Lower dilutions of sucrose fluids of No. 6 strain were too opaque for satisfactory observations; sucrose fluids of B512-F strain were comparatively clear and suitable for observation even when undiluted; solutions (0.1%) of purified dextrans of these 2 strains also show this difference in opacity.

an "intermediate" strain of type 2 pneumococcus, which, although not having a serologically demonstrable capsule, did possess sufficient of the type specific capsular polysaccharide to produce antiserums which reacted with solutions of type 2 S and which also gave Quellung reactions with the cells of fully encapsulated strains of type 2 pneumococcus.

Reactive soluble substances in leuconostoc cultures. The results of tests of supernatant fluids of broth cultures from which the bacterial cells had been removed by centrifugation are presented in Table V. It is evident from the reactions with the types 27 and 32 antiserums that the non-dextran antigens were contained as free soluble substances in the culture fluids of both the leuconostoc strains, but in definitely greater amount in the case of the encapsulated B512-F strain than in the case of the No. 6 strain that had no demonstrable capsule. In contrast, the apparent amount of dextran in the sucrose culture fluids (indicated by the tests with the type 2 antiserum) was essentially the same for both the strains. This contrast illustrates the separability of the capacity to synthesize dextran from the capacity to elaborate non-dextran antigens. The same general principle has been demonstrated previously by the lack of obvious difference in dextran production between Carlson's(14) encapsulated strain of *L. dextranicum* and a non-encapsulated variant derived from it; and also by Horsfall's(9) observation of comparable levan production on the part of encapsulated and non-encapsulated forms of *S. salivarius*.

The fact (Table V) that soluble non-dextran antigens accompany the dextran in the fluids of laboratory-grown sucrose broth cultures indicates that some small amount of similar substances are contained in the large scale commercial cultures or enzyme mixtures from which dextran plasma volume extenders are manufactured. However, the treatments included in the manufacturing process which remove such recognized accompanying substances as culture medium constituents and the bacterial cells would also probably remove or destroy the soluble non-dextran leuconostoc antigens; and, in fact, we failed to detect them in any of several samples of clinical dextrans that we examined by routine precipitation tests with type 27 pneumococcal antiserum. Nevertheless, the probable presence of the soluble antigens in the original fluids is worthy of mention, since it is desirable to have knowledge of all the serologically reactive accompanying substances contained in the source material of any product intended for intravenous administration to humans.

Discussion. Leuconostoc bacteria are a heterogeneous group, comparable to the non-hemolytic streptococci of the respiratory tract in respect to serological diversity of the strains included. Although the "types" set up by McCleskey(7) on the basis of reactions with leuconostoc antiserums are an important step in that direction, a definitive serological classification of the leuconostoc group, if attainable at all, is probably far in the future. Under these circumstances, any serological test that can aid in distinguishing likenesses and differences among leuconostoc strains has present practical value. That cross reacting capacities against different types of pneumococcal antiserums would have some use for that purpose is indicated by the prominent and clear cut distinction between the type 27 and the type 32 reactions of the two strains reported in this paper. Certainly, the definition of the medically important B512-F strain would be made more complete and more precise by including its capacity to react with type 27 but not with type 32 pneumococcal antiserum as a part of its differential description. The applicability to the leuconostoc group would be expected to be limited; and, indeed, it is evident from our preliminary survey of a small collection that some strains give no significant cross reactions with any pneumococcal type. However, cross reactions, when properly controlled and when carefully standardized samples of appropriate antiserums are employed, have fundamental inherent advantages in the differentiation of closely related "types" within a bacterial species. For example, as illustrated in Eddy's (15) studies on inter-related types of pneumococci, there are instances in which types or strains can be identified more easily and also more precisely by cross reactions with heterologous antiserums than by reactions with the homologous antiserums. In view of these potential advantages, further studies with a larger collection of strains will be made to get a more adequate idea of the extent of applica bility of antipneumococcal reactions to the differentiation of leuconostoc bacteria.

The Quellung reaction of the B512-F strain of *L. mesenteroides* reported in this paper, together with the Quellung recently reported by Carlson(14), for a strain of *L. dextranicum,* constitute the first serological demonstration of capsules for the *Leuconostoc* genus. These capsules get their serological reactivity from non-dextran constituents, and are distinct from the "slime" and gelatinous or zooglea-like masses in which leuconostoc bacteria may be imbedded when grown in media of high sucrose content. Not all strains of leuconostoc bacteria would have serologically demonstrable capsules; "intermediate" forms comparable to the No. 6 strain studied in the present paper are undoubtedly frequent and probably also are non-encapsulated Rough forms similar to those isolated by Carlson(14) from an encapsulated strain of *L. dextranicum* and by us (16) from the encapsulated B512-F strain of *L. mesenteroides.* Nevertheless, the fact that some strains possess readily demonstrable serologically reactive capsules carries important implications. That is, from analogy with penumococci, the qualitative differences in the non-dextran capsular substances that would occur among different leuconostoc strains which were in the fully encapsulated "S" form or in the "intermediate" form, together with the loss of capacity to elaborate the reactive capsular substances by Rough variants, can be regarded as the chief factors underlying the prominent serological differences that exist within the leuconostoc group. These factors come from non-dextran components and would be operative with leuconostoc grown in any culture medium. However, when grown in sucrose-containing mediums, the cells of some (not all) strains carry some dextran on their surfaces or as a part of their cell structure(17); the serological properties of the cells then comprise a combination of the properties of the dextrans superimposed on the independent properties of the non-dextran antigens. Under those conditions, the differences that exist among the dextrans(1-4) present an additional factor which would contribute to the differences in antigenic composition and in serological reactions that occur among strains of leuconostoc bacteria.

Summary. 1. The experiments dealt with the antipneumococcal cross reactions of non-dextran antigens of two strains of *L. mesenteroides.* The B512-F strain, which is commonly employed in manufacture of dextran plasma volume extenders, gave reciprocal cross reactions with type 27; the other strain gave reciprocal reactions with type 32 pneumococcus. The data proved that these immunological relationships depended upon similarities between the non-dextran antigens of the leuconostoc bacteria and the specific capsular polysaccharides (S) of the cross-reacting types (27 or 32) of pneumococci. 2. The type 27-reactive antigens of the B512-F strain were shown to be capsular constituents by Quellung reactions; this strain also liberated considerable amounts of the reactive material as a soluble substance into the fluids of broth cultures. The No. 6 strain failed to give Quellung reactions both with type 32 and its homologous antiserum, and appeared analogous in serological behavior to "intermediate" strains of pneumococci that have been described by others. 3. The possible use of antipneumococcal cross reactions as an aid in the differentiation of some strains of leuconostoc was suggested, and factors that underlie the serological diversity of the leuconostoc group were discussed.

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Chromogenic Substrates for Aminopeptidase.* (21443)

G. GOMORI.

From the Department of Medicine, University of Chicago.

The titration method usually employed for the determination of peptidase activity is relatively insensitive and cumbersome. For this reason, the development of substrates with a chromogenic moiety, suitable for the colorimetric assay of peptidase activity and, possibly, for its histochemical localization appeared to be desirable. Such substrates for the test tube assay of carboxypeptidase and chymotrypsin have been devised by Wolf and Seligman(1,2); however, the enzymes could not be demonstrated histochemically because they occur in the tissues in the form of inactive precursors(3,4). A chromogenic substrate, hydrolyzed by an unidentified peptidase, and suitable also for its histochemical localization has been reported by Gomori(5).

Aminopeptidases have been studied with the aid of a number of different substrates(5-15), among which some were aromatic(5,9-12) but not significantly chromogenic. In the report which follows the preparation of chromogenic aminopeptides and their hydrolysis by enzymes of human origin is described.

Methods. Glycyl α- and β-naphthylamine were prepared first, as the simplest possible chromogenic aminopeptides. Three g samples of chloroacetyl naphthylamines were dissolved in 100 ml of methanol, and an equal volume of strong ammonium hydroxide was added. The mixtures were kept in a pressure bottle at 37 to 40°C for 24 hr, filtered and then evaporated at the same temperature under moderate vacuum, using an air inlet, to about ¼ of the original volume. Thirty ml of ethanol and enough water to make 100 ml were added. The mixture was acidified with dilute acetic acid and brought to a boil. An almost clear solution was obtained. About 1 g of adsorbent charcoal was stirred in, and the mixture was filtered hot. Enough dilute NaOH was added to the filtrate to make it slightly alkaline to phenolphthalein, and the mixture was placed in the ice cube compartment of the refrigerator. On cooling, shiny white platelets (β) or needles (α) precipitated which were filtered off, washed with ice cold 30% ethanol and dried at 50°C. Yield, about 55% of the α compound and about 25% of the β compound.

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Analysis of both compounds gave identical values (C, 71.2 to 71.8%; H, 5.89 to 6.05%; N, 13.6 to 14.1%), in good agreement with the formula of $H_2NCH_2CONHC_{10}H_7$. The α compound melted at 105°C; the β compound at 114-115°C. Subsequently, α-alanyl-β-naphthylamine and β-alanyl-β-naphthylamine were prepared by an analogous procedure. In the case of the alanyl derivatives, the exchange of halogen for NH_2 was slower, requiring more energetic heating (in the case of the α-alanyl derivative, 50 to 60°C for 24 to 36 hr; in the case of the β-alanyl derivative the reaction was incomplete even after 6 to 8 days). The yield of the latter compound was low (12 to 15%). Melting points: α-alanyl-β-naphthylamine, 99-100°C; β-alanyl-β-naphthylamine, 85-86°C. *Human tissue* extracts were prepared and assayed for enzymatic activity according to a method reported previously(5); additional sources of enzyme were human serum, aspirated duodenal juice and urine.

Results. The data which follow were obtained with glycyl-β-naphthylamine as a substrate unless otherwise specified. *pH optimum.* The pH dependence of the rate of hydrolysis by human renal extract is shown in Table I. All subsequent experiments were run between pH 6.8 and 7.0.

Effect of substrate concentration on rate of hydrolysis. The absolute rate of hydrolysis is proportional to the concentration of the substrate up to about 0.002M beyond which point the curve gradually bends down but does not become horizontal even at 0.01M. In subsequent experiments, the concentration of 0.0005 M (0.1 mg/ml) was chosen arbitrarily. *Proportionality between the amount of enzyme and the extent of hydrolysis.* Between 0.015 and 0.15 μM of substrate hydrolyzed per hr, the extent of hydrolysis is proportional to the amount of enzyme used. *Course of hydrolysis in time.* Hydrolysis is proportional to time at least up to 2½ hr. *Rates of hydrolysis by various human tissues, serum, duodenal juice and urine.* Table II shows the rates of hydrolysis by human kidney, liver, pancreas, spleen and stomach, in terms of μM of naphthylamine liberated per hr and per g of tissue. These rates of hydrolysis form a pattern very different from that reported for chloroacetyl naphthylamine(5).

TABLE I. Hydrolysis of Glycyl-β-naphthylamine by Extracts of Human Kidney Dependence on pH. Acetate (pH 5) or phosphate (pH 6 to 8) buffers. 0.0002M substrate. 1 mg of tissue per tube. Incubation, 1 hr.

pH	5	6	6.35	6.6	6.8	7	7.4	7.7	8
μg of naphthylamine liberated	0	35	46	48	48	49	41	26	12

TABLE II. Hydrolysis of Glycyl-β-naphthylamine by Extracts of Human Tissues. μM of naphthylamine liberated/hr/g of tissue.

Kidney	Liver	Pancreas	Spleen	Stomach
220	3	10	5.5	1.5

Forty-four samples of human serum were tested (1 ml of a 1:10 dilution per tube). The values were in a narrow range, from 0.6 to 1.3 μM/ml/hr. Eighty-six of the 97 human urine samples tested (1 ml of a 1:3 dilution per tube) showed a significant activity (0.05 to 1.4 μM/ml/hr.) The urines from two cases of nephrosis were the most active. No attempt was made to determine the daily excretion of enzyme. Three samples of human duodenal juice (1 ml of 1:50 dilution per tube) ranged from 5.5 to 17 μM/ml/hr in activity. *Inhibition and activation effects.* The effect of the following metals was investigated at a concentration of 0.001M: Ca, Co, Mg and Mn. Mg is without effect; Ca causes an inhibition of about 25%; Mn, about 35%; Co activates to the extent of 30%. The activating effect of Co on aminopeptidases has been reported by several workers(13,15-17). Fluoride (0.001M) and citrate (0.01M) are without effect. *Hydrolysis by other enzymes.* No hydrolysis of glycyl-β-naphthylamine is effected by 1 mg of crystalline chymotrypsin, 4 mg of crystalline pepsin (at pH 6.8 and 2.8), 5 mg of crude trypsin or 10 mg of crude papain. *Relative rates of hydrolysis of the various substrates.* Glycyl-α-naphthylamine is hydrolyzed by human kidney or serum 6 to 8 times slower than the β isomer. α-Alanyl-β-naphthylamine on the other hand, is hydrolyzed 4 to 5 times faster. β-Alanyl-β-naphthylamine or lactyl-β-naphthylamine, prepared according to Bischoff & Walden(18), are not hydrolyzed at a noticeable rate by comparable amounts of any of the human enzymes used in the experiments described. This finding is in line with the known structural specificity requirements of aminopeptidase.

Histochemical localization of enzyme activity. Paraffin sections of acetone fixed human and dog tissues were incubated with α-alanyl-β-naphthylamine as in the case of chloroacetyl naphthylamine(5). Reactions of moderate intensity were obtained after 1 to 2 hr in some tissues (kidney, intestinal mucosa). However, very intense reactions were secured in a matter of minutes with frozen sections of a number of tissues fixed for 12 to 24 hr in neutralized chilled formalin. Unfortunately, the azo dye formed has a tendency to reorganize into a coarse crystalline precipitate after about 30 minutes. For permanent record, microphotographs must be taken as soon as the reaction has attained a sufficient intensity. The use of diazonium salts other than Diazo Garnet GBC (Red B, Red AL, Red RL, Blue B) will delay crystallization but will not prevent it. Among normal tissues the most intense reactions are obtained in the convoluted tubules of the kidney, the biliary ducts of the liver, the epithelium of duodenal villi and certain unidentified connective tissue elements. Several neoplasms, especially a renal carcinoma, also reacted fairly intensely.

Summary. Chromogenic aminopeptides have been synthesized which are readily hydrolyzed by extracts of human tissues and human serum, duodenal juice and urine. It is possible to localize enzymatic activity histochemically by the use of these substrates.

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Effects of Pentobarbital and Ether upon Vasomotor Responses from Cerebral Cortex of the Dog.* (21444)

HERBERT G. LANGFORD, MAXINE BERNHAUT AND EBBE CURTIS HOFF.

From the Departments of Neurological Science and Medicine, Medical College of Virginia, Richmond.

Blood pressure responses from the cerebral cortex of the dog have not been studied as extensively as those evoked by stimulation of the cortex of the cat and the monkey. The reports available are conflicting. In Danielewsky's original paper in 1870, rises of arterial blood pressure were described. However, Crouch and Thompson(1) stated that 4 dogs investigated under pentobarbital anesthesia gave depressor responses. Four more animals

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* Supported by a contract with the Office of Naval Research, Department of the Navy, Washington, D.C.

were operated upon using curare only. These showed an initial depressor response, with a later rise. Kremer(2) consistently obtained depressor responses upon stimulation of the cerebral cortex with a sine-wave electrical current or with acetyl-beta-methylcholine chloride. As a further indication of the action of anesthetics upon the blood pressure responses to cortical stimulation in the dog, the present study affords evidence of differential effects of pentobarbital and ether upon the cortical autonomic response mechanism.

Methods. In a first series, 7 dogs were given 25 mg of pentobarbital per kilo of body weight. Tracheotomy was done 15 minutes later and the animals were maintained on intermittent positive pressure breathing of air. Craniotomy was performed and the left femoral artery cannulated for blood pressure recording with a sylphon-bellows manometer system. D-tubocurarine (Squibb) was administered intramuscularly in successive doses as necessary to block skeletal muscular movement. The cortex was stimulated with a sine-wave current obtained from an inductorium-type stimulator (Phipps and Bird) using threshold stimuli delivered through bipolar platinum electrodes. Each stimulus was of 10 seconds duration. The second series comprised 8 dogs given d-tubocurarine intramuscularly as in the first series, then ether by cone. Fifteen minutes elapsed before intermittent positive pressure breathing was instituted. While ether was given intermittently throughout the experiment in amounts sufficient to abolish the direct corneal reflex, more was given during craniotomy than later. Approximately 350 cc were administered during the average experiment, which lasted 3-5 hours.

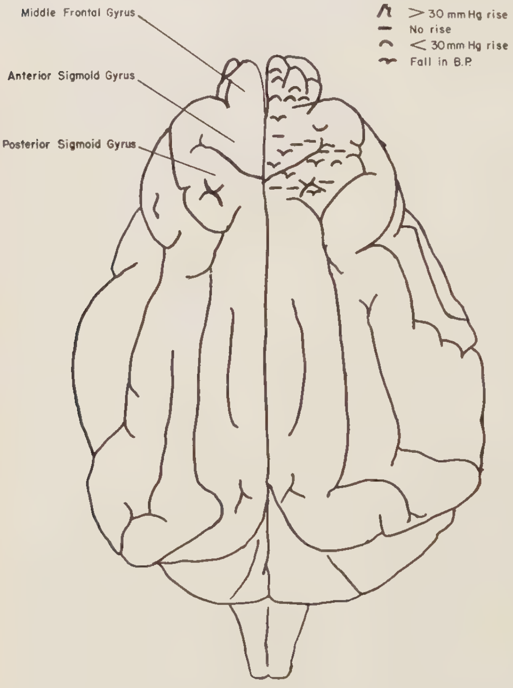

FIG. 1. Blood pressure response from dog anesthetized with pentobarbital. Each symbol represents response to 5 stimulations.

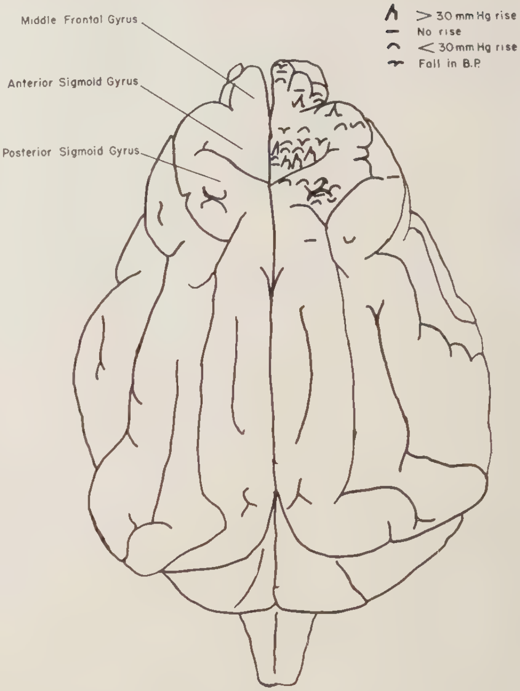

FIG. 2. Blood pressure response from dog anesthetized with ether. Each symbol represents response to 5 stimulations.

Results. No blood pressure response whatever was elicited from 3 animals in series I and they are not charted on the accompanying figure. The results in the 4 remaining dogs with pentobarbital anesthesia are given in Fig. 1. It will be seen that the anterior and posterior sigmoid areas give consistently depressor responses. However, from the most rostral areas a definite pressor response was obtained from nearly all stimuli. It should be noted, however, that these responses were not obtained 4 or 5 hours after the onset of the experiment, so the depth of anesthesia probably was much less by then.

The results of the second series, comprising 8 dogs, are charted in Fig. 2. The response is now predominantly pressor, the most striking changes being obtained from the anterior sigmoid gyrus. The response differs from that of the cat in that many of the blood pressure rises, instead of lasting only 30 seconds, may persist one to 2 minutes, with elevation of blood pressure at times 100 mm Hg above baseline levels.

There was no consistent difference in response in the etherized dogs between the stimuli administered early in each experiment and those administered late, when ether saturation probably was lower.

Also, there was a comparatively small number of stimulations, usually rostrally, to which no response was given for approximately 15 seconds, then a delayed, pronounced pressor response. Exophthalmosis and pupillary dilatation usually occurred with blood pressure rises, but at times there were good pressor responses without exophthalmosis or pupillary dilatation.

Discussion. Striking differences are found in the blood pressure responses evoked upon stimulating the cerebral cortex of the dog anesthetized with pentobarbital, which gives predominantly depressor changes, and the dog anesthetized with ether, in which the arterial blood pressure rises.

An explanation of these differences is not forthcoming. Lindgren(3) has described both vasodilator and vasoconstrictor tracts in the medulla. One might hypothesize that in the animal anesthetized with ether, cortical stimulation fires the vasoconstrictor fibers, while in the animal anesthetized with pentobarbital it activates the vasodilator fibers. We have evidence that the former is true for light ether anesthesia(4).

Of interest also is the frequently much longer duration of blood pressure rise found in the dog than in the cat. Two possibilities should be explored. The most likely cause is continued after-discharge from the cortex. While the response of the cat is little affected by adrenalectomy, it may be that in the dog there is a relatively greater adrenal medullary discharge, with a consequent prolongation of pressor responses.

Summary. 1. Electrical stimulation of the anterior cerebral cortex of the dog under pentobarbital anesthesia gives predominantly depressor responses. 2. Under ether anesthesia the response is usually pressor. Some stimuli are followed by a rise of blood pressure of one of 2 minutes duration, while others are accompanied by only 15 to 20 seconds of pressure elevation. 3. The mode of action of these anesthetics in affecting the cortically induced blood pressure response is not understood.

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Further Observations on Aorta to Coronary Sinus Anastomosis of Beck in Dogs.* (21445)

DAVID S. LEIGHNINGER AND RICHARD W. ECKSTEIN.

From the Departments of Surgery, Medicine, and Physiology, Western Reserve University School of Medicine, Cleveland.

A previous report(1) described late physiologic data up to one year following the aorta-to-coronary sinus anastomosis of Beck (2) in dogs. This procedure for revascularization of the heart is known as the Beck #2 Operation. Coronary artery retrograde flow measurements were made using the Mautz-Gregg method (3). This consists of dissection of the circumflex ramus of the left coronary artery at its origin, ligation of the artery proximally, transection of the artery beyond the ligature, cannulation of the distal end of the artery, and measurement of the flow from the distal circumflex artery in regard to amount, oxygen content and pressure. After the operation the retrograde flow arises from two sources. One source is from the aorta through the graft into the coronary venous system and through a capillary bed where it gives up its oxygen and emerges from the transected circumflex artery as venous blood. The flow from this source is temporary in the dog since it disappears after 5 to 7 weeks. The cause of its disappearance probably is intimal thickening and thrombosis of the veins. The other source of retrograde flow is from intercoronary arterial communications which result from operation. This flow probably is permanent for it exists one year after operation. The retrograde blood, regardless of origin, is highly protective to the heart after a major coronary artery is ligated. This operation reduces mortality and size of infarct after a major coronary artery is ligated(4). Mortality following ligation of the anterior descending ramus of the left coronary artery was reduced from 70% in the normal dogs to 8.9% in 45 dogs 3 to 6 weeks subsequent to the Beck #2 operation. The size of the infarct in the dogs that lived long enough to develop an infarct was reduced by 80% to 90% by the operation and in many specimens there was no gross infarct.

The present studies were to determine 1) if the intercoronary communications in the dog hearts following the Beck #2 operation would persist if the graft were ligated subsequent to the development of the communications, 2) if effective intercoronary communications result from sham operations, and 3) if communications result from other operative manipulations of the heart. This study compares functional intercoronary arterial communications in Beck #2 operated dogs with the following: 1) Beck #2 operated dogs in which the graft was ligated 3 months following the second stage of the Beck #2 operation and studied 3 months following the graft ligation, 2) sham Beck #2 operated dogs, and 3) dogs following defibrillation of the ventricles.

Methods. A. *Beck #2 operated dogs.* The 2-stage Beck #2 operation was performed in 5 dogs. Three to 6 months following the second stage these animals were studied acutely according to the method of Mautz and Gregg(3). Anesthesia was morphine and intravenous pentobarbital. Positive intermittent pressure respiration was used. The heart was exposed through the left chest. The graft between the aorta and the coronary sinus was dissected free so that it could be occluded by clamping. The circumflex ramus of the left coronary artery was ligated near its origin and the distal portion cannulated. The cannula was perfused from the aorta by way of the left common carotid artery. Heparin† was used as an anticoagulant. Mean peripheral circumflex pressure and peripheral circumflex retrograde flow against zero pressure were meas-

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† Part of the heparin was generously supplied by Upjohn Co., Kalamazoo, Mich.

TABLE I. Average Retrograde Flows, Peripheral Circumflex Artery Pressures and Electrocardiographic Evidence of Protection against Circumflex Artery Occlusion in the Group of Dogs Studied.

Condition of dogs	No. dogs	Avg retrograde flow, cc/min.	Range	Avg mean peripheral coronary artery pressure, mm Hg	Range	Evidence of protection, electrocardiographic Protected	Not protected
A. Beck #2 operated	26	11.0*	3.2–33.0	24.9	12–37	20	6
B. Persistence of intercoronary communications	8	9.6*	2.9–20.8	22.7	15–26.5	7	1
C. Sham Beck #2 operated	10	6.9*	.6–16.0	17.4	7–28	8	2
D. Defibrillated	9	4.3	1.5–10.7	17.0	8–29	3	6
E. Non-operated controls	41	3.8	.8–21.0	14.7	7–29	11	25

* Differ from control at 5% level.

ured with the mean aortic pressure maintained at 100 mm Hg. Measurements were made with the graft open and clamped. The electrocardiogram, using lead AVR, was recorded and the effects of circumflex artery occlusions were graded as 0, +, 2+, 3+, or 4+ as previously reported(1). This method of study provides a quantitative evaluation of the functional intercoronary arterial communications in dogs. Simultaneous blood samples were drawn from the aorta and the peripheral circumflex artery with the graft open and clamped. The oxygen content was determined by the method of Neill and Van Slyke. At the end of the experiment, the ostium of the sinus was occluded and a thermal setting plastic was injected into the graft. The tissues were corroded leaving a cast of that portion of the coronary sinus system of veins remaining patent. The degree of venous filling was noted. B. *Persistence of intercoronary communications.* The Beck #2 operation was done in 8 dogs. Three months following the second stage the graft was ligated. Three months following the graft ligation the dogs were studied as in A. C. *Sham Beck #2 operated dogs.* A sham Beck #2 operation was done in 10 dogs. A free graft of jugular vein was anastomosed between the aorta and the coronary sinus. The graft was ligated before the clamps were removed from the aorta and sinus. No blood flowed through the graft at any time. Three weeks later a ligature was placed around the ostium of the coronary sinus and the ligature loosely tied so as not to restrict sinus flow. The knot was then cut out and the ligature removed. Four to 5 weeks later these animals were studied as in A. D. *Defibrillation dogs.* Nine dogs that had been used to demonstrate the technic of cardiac resuscitation were studied. In these dogs the heart had been widely exposed and the ventricles fibrillated with an electric shock. The heart was allowed to fibrillate for 2 minutes. The heart was then massaged and a normal cardiac rhythm restored by means of an electric countershock(5). Four to 52 weeks later the dogs were studied as in A.

Results. A. *Beck #2 operated dogs.* The findings in these 5 dogs were similar to studies previously reported. There was no difference

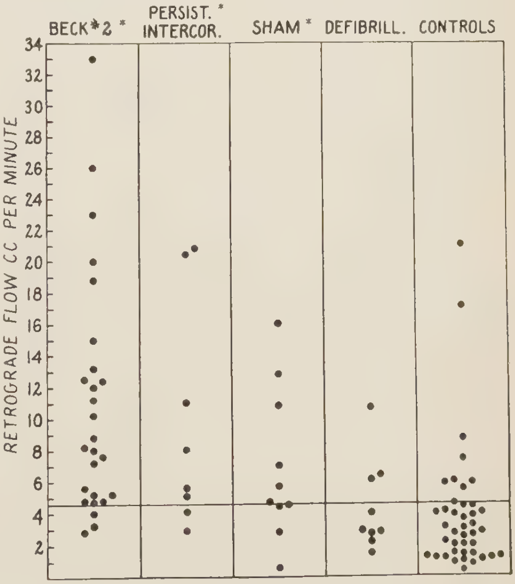

FIG. 1. Retrograde flow for each dog in the group studied. Each dot represents flow for one dog. Horizontal line drawn through median of combined groups.

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* Differ from control at 5% level.

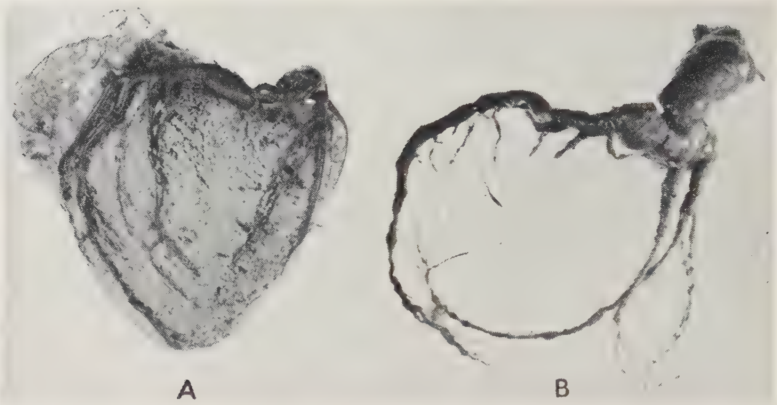

FIG. 2. Plastic cast of coronary sinus system in dog hearts. A. Normal. B. Six months following second stage of a Beck #2 operation.

between the oxygen content of the aortic and retrograde circumflex artery blood either with the graft open or clamped. Because of the similarity of findings in these animals to the Beck #2 operated dogs, studied in the same general time period following operation and previously reported(1), the 2 series have been combined in Table I A and Fig. 1. Seven dogs studied before the graft had lost contact with the capillary bed and previously reported have been deleted from the table and figure. The data in Table I A and Fig. 1 were obtained from dogs studied between 4 weeks and one year subsequent to the second stage of the operation and were obtained with the graft clamped since there was no significant difference between the values with the graft open and clamped. Table I E and Fig. 1 show the data obtained from 41 normal control dogs(6).

The average retrograde flow in the Beck #2 operated dogs is significantly different from that in the control dogs. Twenty of the 26 Beck #2 operated dogs showed electrocardiographic evidence of protection against the effects of circumflex artery occlusion. This is a significant increase over the control group in which 11 of 36 dogs showed similar protection.

Inspection of the 5 Beck #2 operated dog hearts in which the graft and sinus system were injected with plastic followed by corrosion showed that in 4 of the hearts only the major venous branches were filled with the plastic (Fig. 2B). The remaining heart showed filling of very small vessels similar to that seen in normal hearts (Fig. 2A) except for the branches that originated directly opposite the site of the sinus anastomosis. In this heart the anastomosis stoma was exceptionally small and measured about one mm in diameter. It is possible that this size stoma did not permit significant elevation of the coronary sinus pressure during life. B. *Persistence of intercoronary communications.* These 8 dogs maintained a significantly elevated average retrograde flow, Table I B and Fig. 1, even though the graft had been ligated 3 months prior to study. C. *Sham Beck #2 operated dogs.* The data obtained from these 10 dogs are shown in Table I C and Fig. 1. The average retrograde flow is significantly different from the control dogs. D. *Defibrillation dogs.* The data obtained from these 9 dogs are shown in Table I D and Fig. 1. These data are not significantly different from the non-operated control dogs.

Discussion. The observations on the hearts of 5 additional Beck #2 operated dogs are similar to previous data and support the conclusions that the chronic protection of the Beck #2 operation in dogs lies in the development of intercoronary arterial communications and that the perfused venous system does not maintain functional contact with a capillary bed to allow retrograde perfusion of the myocardium. That the block is anatomical as well as functional is shown by the vascular pattern found in 4 of the 5 injected and corroded specimens.

The observations on the hearts of dogs

studied 3 months after the graft had been ligated show that the intercoronary arterial communications developed by the Beck #2 operation persisted for this length of time even though the graft had been ligated. The previous demonstration of functional intercoronary communications in dogs studied one year after the Beck #2 operation, and therefore about 10 months after the grafts had lost functional contact with a capillary bed, also supports the finding that the intercoronary arterial communications in the Beck #2 operated dogs are permanent.

The authors had suggested(1) previously that a stimulus to the production of the intercoronary communications was anoxia. In the present studies, however, this stimulus had been presumably removed by ligation of the graft. The fact that the intercoronary communications persisted in these dogs is interesting in view of the evidence obtained in anemic dogs. Preliminary studies in dogs made chronically anemic by repeated bleeding indicate that intercoronary arterial communications develop but probably disappear following correction of the anemia(7). Additional evidence that the stimulus to the production of intercoronary arterial communications was not anoxia was obtained from the sham Beck #2 operated dogs in which significant collateral growth resulted from surgical manipulation in the absence of myocardial anoxia. Beck has shown(8) that inflammation was a stimulus to the production of intercoronary communications. The Beck #1 operation utilizes this stimulus. Inflammation from operative manipulation of the heart may explain the development of intercoronary communications in the sham Beck #2 operated dogs.

It has been the impression of some investigators that simple exposure of the heart by opening the pericardium may later result in a lower mortality subsequent to coronary artery ligation. A consideration of the data obtained from the defibrillated dogs indicates that even if protection does exist it does not depend upon the development of intercoronary communications.

Summary. 1. Measurements of retrograde circumflex artery flow, peripheral coronary artery pressure, and electrocardiographic changes induced by circumflex artery ligation were evaluated in an additional 5 Beck #2 operated dogs. 2. Twenty-six Beck #2 operated dogs studied from 4 weeks to one year after the second stage of the operation have been compared with: 1) 41 control dogs, 2) sham Beck #2 operated dogs, 3) defibrillated dogs, and 4) Beck #2 operated dogs 3 months after ligation of the graft. 3. Beck #2 operation results in a significant development of intercoronary arterial communications which persist for at least one year in the presence of a functioning graft and for at least 3 months after ligation of the graft. 4. Sham Beck #2 operated dogs develop intercoronary arterial communications which is in contrast to the lack of such communications following ventricular defibrillation. 5. Further substantiation is provided for previous evidence that in a majority of the Beck #2 operated dogs both functional and anatomical contacts between the perfused venous system and a capillary bed are eventually lost. Consequently earlier conclusions are supported that after about 5 weeks the Beck #2 operation protects normal dogs from the effects of coronary artery ligation by means of intercoronary arterial communications.

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Azide Inhibition of Anthrone Colorimetric Test for Carbohydrate.* (21446)

Harold A. Levey.† (Introduced by C. M. Szego)
From the Department of Zoology, University of California, Los Angeles.

In the course of studies on the *in vitro* utilization of carbohydrate by various tissues, the observation was made that when the incubation medium contained sodium azide (10^{-2}-10^{-3} M) as well as glucose (100 mg %), none of the latter could be detected by the anthrone method of Durham *et al.*(1). Since it was unlikely that azide promoted utilization of all the glucose, the possibility appeared that azide interfered with the condensation of anthrone and the sulfuric acid dehydration product of glucose (considered to be a furfural derivative (2)). The present study was designed to determine the effect of azide on various colorimetric methods for the determination of carbohydrate.

Methods. The anthrone method for total carbohydrate(1), the ferricyanide method for reducing sugar(3), and the skatole method for fructose(4) were performed in the presence and absence of sodium azide as indicated in Table I.

Results. It can be seen in Table I that 5 x 10^{-3} M azide totally inhibited anthrone color development with as much as 500 μg of glucose. Inhibition was also seen with fructose in the test solution. However, 10-fold dilution of the azide allowed recovery of about half of a 50 μg test sample of glucose. The ferricyanide method for reducing sugar and the skatole method for fructose were unaffected by azide.

Discussion. While the mechanism of the anthrone reaction is not fully understood, the suggestion has been made(2) that a colored product is formed by the condensation of anthrone with a furfural derivative of hexose, the latter substance being produced by the de-

TABLE I. The Effect of Azide on 3 Colorimetric Tests for Carbohydrate.

Carbohydrate in test solution†	Molar conc. of sodium azide	μg carbohydrate recovered by each method* — Anthrone method for total carbohydrate	Ferricyanide method for reducing sugar	Skatole method for fructose
μg μg				
50 G	0	50		
50 F	0	47		
25 G + 25 F	0	45.2		
50 G	5 × 10^{-3}	0		
50 F	”	0		
25 G + 25 F	”	0		
200 G	”	0		
500 G	”	0		
50 G	5 × 10^{-4}	28		
10 G	0	10	10	
10 F	0	10.4	10.4	
5 G + 5 F	0	10.3	10.6	
10 G	1 × 10^{-3}	0	10.4	
10 F	”	0	11.4	
5 G + 5 F	”	0	10.9	
20 F	0			20
20 F	1 × 10^{-2}			19.9

* Analyses were performed on 2 ml of solution containing carbohydrate or carbohydrate + azide.

† G = glucose; F = fructose.

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† Present address: Department of Physiological Chemistry, School of Medicine, Medical Center, University of California, Los Angeles.

hydrating action of concentrated sulfuric acid. Azide inhibition of the anthrone reaction might occur by at least 3 mechanisms:

1. "Inactivation" of hexose by direct combination with azide. If such a reaction took place it would probably occur at the aldehyde grouping of the hexose. That it does not occur is suggested by the total recovery of reducing activity of the hexose regardless of the presence of azide.

2. Preferential combination of the furfural derivative of hexose with azide, rather than with anthrone. Both glucose and fructose yield furfural derivatives upon treatment with sulfuric acid. The formation of the furfural derivative of fructose is a step common to both the anthrone and skatole reactions with this hexose. Since the latter reaction was not inhibited by azide, it is unlikely that combination of azide with the furfural derivative underlies the inhibition of the former reaction.

3. Azide reaction with anthrone. In the presence of sulfuric acid, azide is highly reactive with aromatic carbonyl groups (as present in the anthrone molecule) through the mechanism of the Schmidt reaction(5). Since anthrone is present in great excess, there would have to occur preferential condensation of the furfural derivative of hexose with the azide-anthrone product of the Schmidt reaction to yield a colorless complex.

Although only the last possibility is compatible with all the observations in the present study, the actual mechanism of azide inhibition is completely unknown. However, it is obvious that the anthrone method is unsuitable for the determination of carbohydrate in metabolic experiments involving the use of azide.

Summary. It has been demonstrated that azide inhibits the anthrone reaction for the determination of total carbohydrate, but does not affect the ferricyanide test for reducing sugar or the skatole test for fructose. The mechanism of inhibition is unknown.

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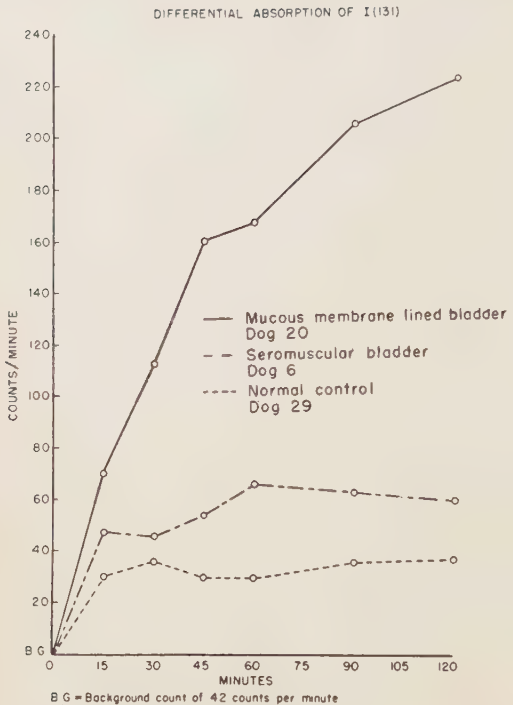
Permeability of Normal and Artificially Constructed Canine Urinary Bladders to I^{131}, Na^{22} and P^{32}. (21447)

HORACE D. MARUCCI, WILLIAM C. SHOEMAKER, ARTHUR W. WASE, HERBERT D. STRAUSS, AND SAMUEL V. GEYER. (Introduced by M. J. Boyd)

From the Departments of Surgery, Urology, Biochemistry and Radiology, Hahnemann Medical College and Hospital of Philadelphia, Pa.

The problem of ion transportation through the mucosa of reconstructed bladders assumes major importance with the increasing number and types of reconstructive procedures on the urinary bladder. The resorption of electrolytes and nitrogenous wastes through the mucosa of artificially constructed bladders with resultant hyperchloremic acidosis and uremia has been a cause of morbidity and mortality in the late post-operative course of patients subjected to cystectomy for bladder tumors.

The present study undertakes to describe a method for the investigation of ion permeability in normal and artificially constructed canine urinary bladders by means of radioactive elements. The absorption patterns of normal canine bladder mucosa were compared with surgically constructed bladders lined with ileal mucous membrane, and with seromuscular grafts in which the transitional cell epithelium was allowed to overgrow the graft(1,2). The availability of radioactive isotopes of iodine



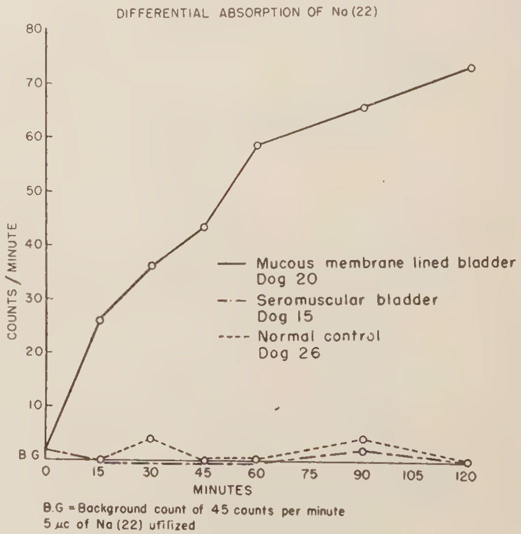
B G = Background count of 42 counts per minute
25 μc of I(131) utilized

FIG. 1.

(I^{131}), sodium (Na^{22}), and phosphorus (P^{32})* reduces the quantitative aspects of the determinations to simple terms, permitting assay of minute amounts of substances without altering the physiological levels of these ions in the test animals.

Materials and methods. Adult mongrel male and female dogs weighing 12 to 15 kg were used. Artificial bladders were constructed by means of technics previously described(1,2) using: (1) segments of ileum with intact mucous membrane, (2) seromuscular grafts which were found by biopsy and necropsy to have been epithelialized with a transitional cell lining. Dogs of similar weight with normal bladders were used as controls. All animals were prepared in an identical fashion. Food was withheld for 12-24 hours and the dogs were then anesthetized with intravenous veterinary sodium pentobarbital. The male animals were catheterized with No. 7 ureteral catheters, and the females with No. 14 Foley catheters. The bladders were then washed with sterile water and evacuated. Control blood and urine samples were taken on each animal at the beginning of the experiment. Thirty-one studies were made in the following distribution: thirteen dogs utilizing I^{131}; 9 dogs utilizing Na^{22} and 9 dogs using P^{32}. For the I^{131} experiments, 25 μc were used; for the Na^{22} studies 5 μc were used, and for the P^{32} 50 μc were used. In each experiment the selected isotope was diluted in 20 ml of isotonic saline solution and instilled into the bladder. Following this, 10 ml of sterile water were introduced into the catheter to clear it of radioactive material. All isotopes used were carrier-free. Blood samples of 5 to 8 cc were taken at 15, 30, 45, 60, 90 and 120 minutes. All *radioactive assays* were performed on 1 ml aliquots of plasma evaporated to dryness on iron planchetts of 2 cm diameter. A thin window (2.1 mg/cm^2) Geiger-Mueller counter was employed with a conventional scaler. The over-all efficiency of the system was 5%. Since all of the isotopes employed are hard emitters, corrections for self-absorption were unnecessary. All counting was performed on the same day for each experiment, and under the same conditions. Counting was continued to give a maximum statistical error of 5%.

Results. The experimental results of 5 normal dogs subjected to the previously described method using I^{131} revealed that only minimal



B.G = Background count of 45 counts per minute
5 μc of Na (22) utilized

FIG. 2.

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* Obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.

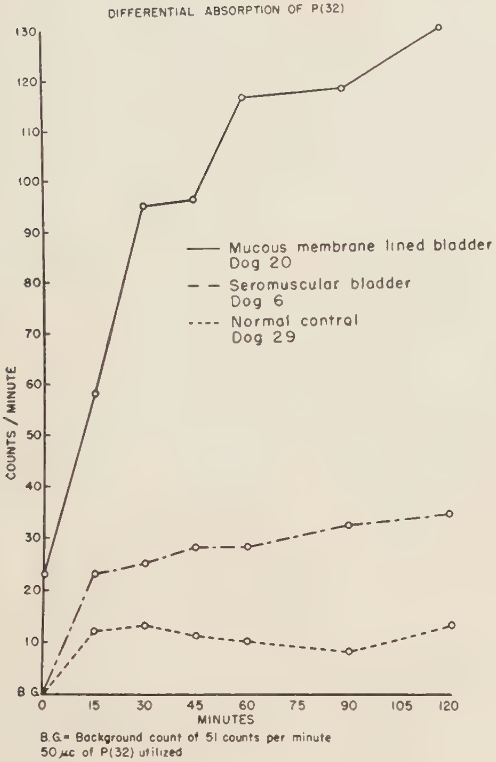

FIG. 3.

quantities of iodide ion were transported across the mucosa. Each of the 4 animals whose bladders were constructed with seromuscular grafts gave comparable findings. However, all of the 4 animals whose bladders were replaced with ileal segments with intact mucous membrane showed rapid absorption of radioiodine with continually increasing peripheral blood levels over the 2-hour period of observation. Fig. 1 shows representative data from each of the 3 types of bladders studied.

Similar ion transportation patterns to I^{131} resulted when P^{32} and Na^{22} were used in each of the 3 ileal mucous membrane-lined bladders. Three normal and 3 seromuscular constructed bladders consistently showed minimal P^{32} ion transportation, while the same number of animals showed only traces of Na^{22} ion absorption over the test period. Fig. 2 and 3 show representative Na^{22} and P^{32} ion transportation patterns on each of the three types of bladders.

Summary. A method for determining ion transportation through the mucosa of the urinary bladder is described. This method has been used on normal and artificially constructed bladders in dogs.

Rapid and continuous absorption of iodide, sodium and phosphorus ions takes place through the mucous membrane lined bladders, while only minimal amounts of these ions are absorbed through the normal bladder and through bladders constructed by means of seromuscular grafts.

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Effect of Certain 2,4-Diaminopyrimidine Antagonists of Folic Acid on Pregnancy and Rat Fetus.* (21448)

J. B. THIERSCH (Introduced by F. S. Philips)

From the University of Washington School of Medicine, Seattle.

Hitchings and collaborators(6) synthesized 2, 4-diamino-5-p-chlorophenyl-6-ethylpyrimidine(50-63) and 2, 4-diamino-5-(3′, 4′-dichlorophenyl) - 6 - methylpyrimidine(50-197). The compounds resembled in their biological action the 4-amino analogues of folic acid(11). They showed also a marked activity in small doses, against experimental and human malaria(4) and experimental animal tumors(2,3,11). Previous work with 4-aminopteroylglutamic acid (4-amino-PGA) had shown that this analogue, when given in small quantities, would induce rapid death of the

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implanted early embryo of mice, rats(13) and man(14). Other workers had also demonstrated that the withdrawal of folic acid from the diet or the feeding of the mild antagonist, x-methylfolic acid, induced a great variety of malformations in the growing fetus, and that the antagonist, if given in sufficient quantity, would lead to fetal death and resorption(5,10). The present study was initiated to determine whether the 2, 4-diaminopyrimidines, 50-63 and 50-197, could affect the developing rat fetus in a manner similar to other folic acid antagonists.

Procedure. Experiments were conducted with the Long-Evans strain of rats, bred and reared in our own colony. Animals were maintained on the "White" breeding diet of Long and Evans* *ad lib.* For controls and experimental work only rats that had at least one previous litter were used to avoid irregularities of the first gestation. The animals weighed between 250 and 300 g and were 7 to 9 months old. The gestation period of this strain is 22 days. Considering the morning of massive sperm findings in the vagina as day zero, implantation of fertilized ova takes place on the seventh day(9). The average number of implantations for this strain of rats is 8.6. The average weight gain of non-pregnant rats for a period of 21 days was 8%, of pregnant rats receiving tap water or placebos, 36%. Fertilized animals were kept in single cages and periodically weighed and treated. The experimental and control rats received the drug or a placebo in a suspension of 10 cc of water per kg body weight administered with the stomach tube. Struggling of the rats and spilling were carefully avoided and only a single rat dose was drawn up in the syringe at a time, to prevent over-dosing. The controls consisted of one group of rats (Control B, Table I) treated with tap water from the 5th to the 14th day and another group (Control A, Table I) treated from the 7th to the 16th day of gestation with an ineffective compound, 2-acetylamino-1-(3′,4′-thiadiazole)-5-sulfonamide (Diamox, Lederle). The control rats were in all respects handled and treated like the experimental rats but kept in a separate room. All rats were sacrificed under ether anaesthesia on the 21st day of gestation, the day prior to expected littering. Maternal hemoglobin and sternal bone marrow were examined, the uteri removed intact, and implantations counted. The uteri were then opened longitudinally, the fetuses counted, measured and weighed, inspected for malformations, and the head cross-sectioned to detect internal hydrocephalus. All stunted malformed fetuses, as well as implantation sites without fetuses, were sectioned, x-rayed, and fetal hemoglobin determined. Originally each experimental and control group was started with 30 rats. Of these approximately 70% were found to have been or to be pregnant at the time of sacrifice.

Results. Effect of 50-197. The LD_{50} for Wistar rats is 5 successive intraperitoneal doses of 7 mg/kg/day(15). Several doses of 5 mg/kg/day have the same effect in mice (16). Early experiments with 3 doses of 7 mg/kg/day, 2 and 3 doses of 5 mg/kg/day given prior to or immediately following implantation, showed these doses to be toxic and to lead to weight loss in the mother animals (Table I, 7-10). The numerous deaths prior to the day of sacrifice reduced greatly the number of experimental animals. Implantation of the ova was not prevented with this dosage but all the fetuses surviving the medication were found to be arrested in their development. The majority of the implanted ova had become necrotic and were resorbed. The erythro- and myelopoiesis of bone marrow of the experimental animals were severely affected when examined 5 days after the last medication, not more than 30% to 50% of normal bone marrow being present. The dosage was therefore abandoned and further experiments with 2.5 mg/kg/day carried out. The experiments with 1 to 3 doses of 2.5 mg/kg are listed in Table I, 1-6. In all 6 experiments a normal number of implantations was obtained. Three doses given on the 4th, 5th and 6th days of gestation did not interfere

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* "White" diet:	%
Ground whole wheat	67.3
Casein, tech.	15.0
Skim milk powder	7.5
NaCl crystalized	.75
$CaCO_3$	1.5
Melted fat	6.75
Fish oil (vit. A-D conc.)	1.0
K.I. sol., 450 mg/liter	.2
Lettuce supplement twice weekly	

TABLE I. Effect of 2,4-Diamino-5-(3′4′-Dichlorophenyl)-6-Methylpyrimidine on Litter of Rats.

			Mother rat at sacrifice		Fate of litters								
						Fetuses							
No. of rats		Days of treatment	% wt gain 1/21 day of gestation	% normal bone marrow present	Total implants	Live	Stunted	Gross malform.	Dead	Resorb.	Mean litter wt in g	Mean fetal wt in g	Mean fetal length in cm
Control A													
37	a	7–16	+36.1	70	352	349	—	—	—	3	44.6	4.7	4.4
	b				9.5	9.4				.1			
	c					99.2				.9			
Control B													
30	a	5–14	+36.7	”	282	277	—	—	—	5	38.8	4.2	4.0
	b				9.4	9.3				.2			
	c					98.3				1.7			
Experimental													
21	a	5 & 6†	+ 8.1	”	117	72	7	1	3	45	16.0	4.0	4.0
	b				8.1	3.5	.3	.5		2.1			
	c					61.3	d 9.7	d 1.4		38.5			
16*	a	4, 5, 6†	+ 6.4	”	145	55	37	—	—	90	5.1	0.7	0.7
	b				9.1	3.4	2.3			5.6			
	c					38.5	d67.2			62.0			
26	a	7 †	+23.3	80	216	202	35	2	6	14	35.8	4.4	4.4
	b				8.3	7.8	1.4	.8		.5			
	c					93.5	d17.3	d 1.0		6.5			
19	a	8 & 9†	− 5.5	70	116	1	—	1	—	115	2.3	2.3	2.3
	b				6.1	.5		.5		6.0			
	c					.6		d100.0		99.0			
17	a	7 & 8‡	+17.1	”	145	129	29	11	3	9	29.2	3.8	3.7
	b				8.5	7.6	1.7	.7		.5			
	c					89.0	d22.5	d 8.5		7.0			
11	a	6, 7, 8†	+ 5.0	”	85	5	—	5	—	80	2.7	1.6	1.6
	b				7.7	.6		.6		7.3			
	c					.5		d100.0		94.2			
12	a	5 & 6§	− .4	50	96	—	—	—	—	96			
	b				8.0					8.0			
	c									100.0			
20	a	7, 8, 9§	−13.4	”	158	—	—	—	—	158			
	b				7.9					7.9			
	c									100.0			
18*	a	3, 4, 5‖	−18.5	30	4	—	—	—	—	4			
	b				.2					.2			
	c									100.0			
22	a	7, 8, 9‖	−15.8	”	182	15	15	—	—	167	3.2	.4	.5
	b				8.4	.7	.7			7.6			
	c					8.3	d100.0			91.8			

a = Total No. b = Per animal. c = %. d = Of live fetuses.

* Sacrificed on 14th day. † Daily dose, 2.5 mg/kg. ‡ Daily dose, 2.5 mg/kg plus leucovorin. § Daily dose, 5.0 mg/kg. ‖ Daily dose, 7.0 mg/kg.

with implantation, resulted in 38.5% live fetuses of all fetuses present, but over 67% of the live fetuses were stunted. Over 60% of the total implanted ova were completely resorbed (Table I, 2). If only 2 doses were given on the 5th and 6th days of pregnancy 61% of all the implanted ova were found to be alive, of which only 9.7% were stunted, one fetus displaying a gross malformation of the cranium. Three fetuses were found dead and macerated. 38.5% of all fetuses were completely resorbed, reducing the total number of damaged fetuses to 44.7% (Table I, 1). If only one dose of the compound was given on the 7th day or the day of implantation, 93.5% of the implanted ova were found to be alive. Of these, 17.3% were stunted and an additional 2 showed gross malformations of the cranium with hydrocephalus. Only 6.5% of all the implants were resorbed, reducing the total number of damaged fetuses to 23.6% (Table I, 3). Two doses given on the 8th and 9th days of pregnancy resulted in damage to all the implanted ova. Only one live but malformed fetus was detected; all the other fetuses were resorbed (Table I, 4). Three doses given on the 6th, 7th and 8th days of pregnancy showed the same effect, only 5 malformed fetuses with cranial defects being discovered at sacrifice (Table I, 6).

An attempt was made to counteract the action of the drug with leucovorin. For this purpose a group of animals received 3 doses of 10 mg/kg of the calcium salt of leucovorin. The leucovorin was given intraperitoneally on the 7th and 8th days 20 minutes prior to the first and second, and again 12 hours after the second, dose of the pyrimidine. The result is listed in Table I, 5 and shows the striking effect of leucovorin. The mother rats gained weight and 89% of all fetuses were found to be alive. Over 20% of the live fetuses, however, were stunted; over 8.5% of all the live fetuses showed gross malformations. The remainder of the fetuses were resorbed. The malformed and stunted animals measured less than 3 cm in length, showed abnormalities of the cranium, hydrocephalus, ventral hernia, general edema, and hydrops. The total number of damaged fetuses amounted to 33.8% in this group. (Table I, 5).

Microscopic findings, 50-197. All implantation sites were sectioned and carefully studied for the presence of fetuses. Rats treated with 5 or 7 mg/kg showed only a total of 15 fetuses. The implanted fetuses had ceased to develop further. On the 14th day of gestation they corresponded to 8-day embryos. All other fetuses were resorbed, but placenta and decidua could be recognized. The uterine contents of the rats treated prior to implantation with 2.5 mg/kg showed stunted litters which were by comparison with controls approximately one week behind in their development at the time of sacrifice. The malformed fetus had an open cranium, the brain having eroded on the surface, not being covered by meninges or cranial bones. The animals injected during implantation with the 2.5 mg dose showed again stunting with general under-development of internal organs as well as limbs. The malformations consisted of internal hydrocephalus, under-development of cranial bones, microcephalus, ventral hernia, and failure of the cranium to close over the brain. The malformations observed in the group also treated with leucovorin showed great variation. Microcephalus, anencephalus, rachischisis, lack of development of facial structure as well as brain, hydrocephalus, ventral hernias as well as general hydrops, were seen, as well as one case of situs inversus. Doses of 2.5 mg given prior to implantation did not prevent implantation but resulted in a high percentage of resorbed or stunted fetuses. The same doses during implantation led to the rapid death of most fetuses. The few surviving fetuses showed gross malformations. The effect of the drug was greatly reduced but could not be prevented with 6 times the quantity of leucovorin. No undamaged live fetuses were found in any group of mother animals treated with this compound gaining less than 5% of their initial body weight during gestation.

Effect of 50-63. Following the experience with the compound described above, experiments were conducted with 2, 4-diamino-5-*p*-chlorophenyl-6-ethlypyrimidine. According to the available data(15,16), the compound was 10 to 20 times less active in the experimental animals than the methylpyrimidine derivative. Doses of 25 mg/kg induced convulsions and

TABLE II. Effect of 2,4-Diamino-5-p-chlorophenyl-6-ethylpyrimidine on Litter of Rats.

				Mother rat at sacrifice				Fate of litters						
									Fetuses					
No. of rats		Days of treatment	Daily dose in mg/kg	% wt gain 1/21 day of gestation	% normal bone marrow present	% Hgb.	WBC	Total implants	Live	Stunted	Re-sorbed	Mean litter wt in g	Mean fetal wt in g	Mean fetal length in cm
Control A														
37	a	7–16		+36.1	70	12.6	12.6	352	349	—	3	44.6	4.7	4.4
	b							9.5	9.4		.1			
	c								99.2		.9			
Control B														
30	a	5–14		+36.7	”	12.6	12.1	282	277	—	5	38.8	4.2	4.0
	b							9.4	9.3		.2			
	c								98.3		1.7			
Experimental														
14	a	7–16	.3	+21.7	”	12.6	12.3	117	111	3	7	28.0	4.0	3.8
	b							8.4	8.3	.3	.5			
	c								95.0	d 2.7	6.0			
13	a	4–13	.5	+37.2	”	12.7	12.3	113	101	7	12	31.3	4.0	3.6
	b							8.7	7.8	.5	.9			
	c								77.7	d 6.9	8.5			
9	a	”	.5*	+37.1	”	12.9	12.4	71	69	—	2	28.2	3.7	3.7
	b							7.9	7.7		.2			
	c								97.2		2.8			
12	a	”	1.0	+34.3	”	11.0	12.6	120	102	17	18	32.2	3.8	3.7
	b							10.0	8.5	1.4	1.5			
	c								85.0	d16.6	15.0			
24	a	7 & 8	12.5	+21.5	”	11.9	12.3	190	127	16	63	24.0	3.7	3.8
	b							7.9	5.3	.7	2.6			
	c								66.8	d12.6	33.2			
23	a	7, 8, 9	12.5	+ 8.6	60	12.1	12.7	195	61	20	128	22.8	3.7	3.8
	b							8.5	2.7	.9	5.6			
	c								31.3	d32.8	66.2			

* Plus 50 mg/kg PGA.

a = Total No. b = Per animal. c = %. d = Of live fetuses.

death in the experimental rats and were abandoned. Three doses of 12.5 mg/kg given on the 7th, 8th and 9th day of gestation resulted in 31% of live fetuses of which one-third were stunted. Three fetuses were found dead and macerated; all the others were found completely resorbed (66%) (Table II, 6). Two doses of 12.5 mg/kg given at the time of implantation (7th and 8th day) permitted 66% of the fetuses to survive, of which 12% were stunted. One macerated dead fetus was found and 33% of all fetuses were resorbed (Table II, 5). Ten doses of 0.5 mg/kg (Table II, 2) or 1 mg/kg (Table II, 4) given from the 4th through the 13th day of pregnancy and 0.3 mg/kg given from the 7th through the 16th day of pregnancy still had some effect on the developing fetuses (Table II, 1). The 1.0 mg/kg doses induced 15% resorption, the 0.5 mg/kg produced 8.5% resorption and the 0.3 mg/kg dose still 6% resorption, with the appearance of the number of stunted animals decreasing with the diminishing dosage to 2%. The weight gain of the mothers was normal when the compound was administered during the first trimester of gestation, but lagged considerably when the compound was given in the second trimester. Ten doses of 50 mg/kg of folic acid given 30 minutes prior to 10 daily doses of 0.5 mg/kg 50-63 counteracted the effect of the drug to a minor degree, but could not entirely prevent it. However, stunted animals were not noted in that series (Table II, 3). Compound 50-63 given in doses of 12.5 mg/kg following implantation led to a high percentage of fetal death and resorption and produced stunted and malformed fetuses. The weight curve of the mother animals during gestation showed that live fetuses were obtained only if the weight gain was more than 8%.

Microscopic findings, 50-63. The malformations and stunting observed in this group corresponded to those described for Compound 50-197.

Discussion. In experiments with ova of *Rana pipiens* Bieber *et al.*(1) could show a difference in the action of the compounds examined in the present work and 4-amino-folic acid and 6 mercaptopurine. The pyrimidines did affect earlier stages of embryonic development than the aminated folic acid, which was active at the stage of neurulation. With *Lactobacillus casei*(6,7,8) the compounds were competitive antagonists of folic acid and with *Leuconostoc citrovorum* of folinic acid (7). The growth of *Streptococcus faecalis* (8) was prevented with a molar ratio of the pyrimidine to folic acid of 16:1, but much larger doses were required to suppress growth in the presence of folinic acid, suggesting an interference by the compound with the conversion of folic to folinic acid. Young rats(8) when fed the Compound 50-63 showed stunting of growth, leucopenia, alopecia, and blood and marrow changes seen previously with the 4-amino-PGA. The symptoms could be prevented by simultaneous administration of folinic acid but not of folic acid. Furthermore, 70% alcohol insoluble fraction of liver was more effective in preventing toxic symptoms than folinic acid. In mice, rats, dogs and cats (8,11) the pyrimidines produced changes in peripheral blood, bone marrow and intestinal tract similar to the 4-amino analogs of folic acid(12), and the bone marrow of dogs was shown to contain megaloblasts and chromosomal aberrations. Sarcoma 180 in mice(3) was markedly inhibited by the pyrimidines; so was the Ak. M. strain of leukemia in mice (2,6). It was, however, important to note that only folinic acid in mice protected against the action of 50-197(7).

The findings with pregnancy in rats closely correspond to those found with 4-amino-PGA (13). It is of importance to stress that implantation of the fertilized rat ova was not entirely prevented with the doses used, even when the compound 50-197 was given on the 4th day of pregnancy. The malformations observed corresponded also closely to those reported in experiments with the x-methyl or 4-amino-PGA when given in non-lethal doses (10). The most frequent malformation observed in the present experiment was a failure of the cranium to close over the brain. In several instances the entire cranium was only rudimentarily developed. A general hydrops of all fetuses in the litter was seen thrice, so was a ventral hernia. In one litter a variety of malformations of the cranium and spine, as well as ventral hernia, was seen. Outstanding was the general stunting of many embryos without gross malformations, the fetuses measuring only 1-3 cm in length and not more than 2 to 3 g in weight on the day prior to term. In cases of earlier fetal death and resorption the placenta or placental remnants were clearly detectable, varying in size from a normal organ 1 to .5 cm to a small greenish button .3 to .5 cm in length and .1 to .3 cm in thickness. The presence of these placentas, usually covered or surrounded by hemorrhage, permitted an easy counting of the original implantations.

Compound 50-63 has been introduced into effective malaria therapy. The usual dose is 25 mg taken in a single dose once a week. From our data it is evident that this small dose would be harmless to the fetus. An attempt was made to induce therapeutic abortions in 2 women with this compound during the first month of pregnancy. One 23 year old woman suffering from Hodgkin's disease and one 25 year old woman afflicted with bilateral pulmonary tuberculosis were given 200 mg doses of 50-63 each. This led within minutes to projectile vomiting, the patients returning most of the compound with the vomit. Following this experience attempts to use this compound for this purpose were abandoned, and surgical procedures started. The present experiments showed that neither 50-63 nor 50-197 is suitable to control reproduction because both are too toxic to the mother animal in doses destroying the litter.

Summary. (1) 2, 4-diamino-5(3′,4′-dichlorophenyl)-6-methylpyrimidine when given to pregnant rats in the doses described, prior to implantation, did not prevent implantation, but led to stunting or fetal death following implantation. When the compound was given following implantation a stunting or lethal effect on the fetuses was achieved depending on the amount of drug administered. The effect on the fetuses could be moderated with a higher yield of live litters but also of stunted and malformed animals when the mother rat was given leucovorin at the time of drug administration. (2) 2, 4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine had a similar effect on the fetuses in 5 times higher concentration. (3) The malformations induced were similar to those obtained with structurally closely-related folic acid analogues. They consisted in general stunting of growth, general hydrops, cranial bone defects, incomplete cranial and brain development, rachischisis, internal hydrocephalus, ventral hernias, situs inversus, and a combination of all. No live fetuses were obtained from treated mothers who did not gain more than 8% of total body weight during the period of gestation.

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Thyroxine Depression of Mouse Thyroid Iodide Trap Activity. (21449)

H. J. Lipner,* Billie P. Wagner, and Harold P. Morris.

From the National Cancer Institute, National Institutes of Health, U. S. Public Health Service Department of Health, Education and Welfare, Bethesda, Md.

The development of several lines of transplantable thyroid gland tumors in mice(1,2), and investigations of their functional activities have indicated the need for studies of normal mouse thyroid function. One of the easily identifiable and quantitative changes which occurs in these mouse thyroid tumors, as in most thyroid gland tumors, is in the metabolism of iodine(3,4). Some phases of non-tumor-bearing mouse thyroid function have been studied previously(5-7). Experiments on the depressing effect of thyroxine on the activity of the non-tumor-bearing mouse thyroid iodide trap are presented in this report.

Thyroxine inhibits the hypertrophic and iodide accumulating response of both rat and chick thyroid glands to thyrotrophin (TSH). Thyroxine also inhibits the iodide concentrating mechanism of the rat thyroid to a greater extent than does hypophysectomy(8). The iodide concentrating mechanism of the mouse thyroid appears to function as does that of the rat thyroid, except that this mechanism in the mouse possesses a much greater degree of autonomy, *i.e.* independence of hormonal control.

Methods. C_3H/HeN male mice, obtained from NCI stock and ranging in age from 2 to 5 months, were used in all experiments. The animals were adapted gradually to the NCI pellet diet and then placed on the Morris low iodine diet (77A)(4) or the modified 342 Remington diet[†](9) (designated 277). Diet 77A contained $0.75 \pm .039$ μg and diet 277 $0.034 \pm .001$ μg of iodine per gram of diet. The ratio T/S (I^{131}/100 mg thyroid tissue/I^{131} per 0.1 ml serum) was determined in the manner described by Halmi *et al.* (8).[‡] The radioactivity was measured with a scintillation counter, employing a well type thallium activated NaI crystal(10). All dissections were performed under a dissecting microscope at 9× magnification, and completeness of hypophysectomy was confirmed under the microscope. *DL*-Thyroxine (20 mg) or KI (17 mg) was dissolved in 1 ml of 5 N NaOH, diluted to 500 ml with distilled water and brought to a final volume of one liter after the addition of 9 g of NaCl. Both solutions were frozen in enough 10 ml aliquots to last for the duration of the experiment. A single aliquot of each solution was thawed immediately prior to use, and the unused excess discarded. Thyrotrophin[§] powder was dissolved in distilled water to contain the equivalent of 5 mg of USP reference substance per ml. Aliquots of the solution were distributed into several vials and frozen. An aliquot was thawed just prior to use, and any excess was discarded. Both the thyrotrophin and thyroxine solution were administered subcutaneously.

Results. The daily administration of 2 μg of *DL*-thyroxine for 8 days to a group of 7 male mice was followed by a depression in the activity of the thyroid iodide trap, as indicated by the decrease to 49.0 in the value of the T/S ratio, compared to a control value of 253 (Table I A). That this was probably due to the inhibition of thyrotrophin secretion is indicated by the T/S ratio value of 42.0 following hypophysectomy. Thyroxine administration to hypophysectomized mice results in further depression of the trap activity below the hypophysectomy value. The difference between the means was significant at

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[*] Public Health Service Fellow, National Cancer Inst. Present address: Department of Pathology, Chicago Medical School.

[†] Wilson's whole liver powder replaced the pork liver powder as used by Remington(9).

[‡] The I^{131} was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn., on allocation from the A.E.C.

[§] Kindly supplied by Armour Laboratories, through the courtesy of Dr. I. Bunding. Each vial, lot No. 377157, contained the equivalent of 40 mg USP Reference Substance.

TABLE I. Depression of Thyroid Function.

No. of mice	Treatment	Total length of exp. (days)	Dose of drug* (µg) / Treatment (days)	Avg thyroid wt (mg)	T/S
A. Effect of thyroxine and hypophysectomy (Remington diet 277)					
7	Control	8	0 / 8	2.20 ± .20†	253.0 ± 18.4†
7	Thyroxine	8	2.0/ 8	1.42 ± .04	49.0 ± 5.0
3	Hypophysectomized	20	0 / 8	—	42.0 ± 5.3
4	Hypophysectomized and thyroxine	22	2.0/ 8	—	29.0 ± 5.6
B. Effect of thyroxine and iodide (diet 77A)					
6	Control	28	0 / 0	1.56 ± .16	137.2 ± 5.3
7	"	8	0 / 0	1.54 ± .11	164.9 ± 20.4
5	Thyroxine	11	2.0/ 5	2.20 ± .13	47.9 ± 6.6
7	"	8	2.0/ 8	1.30 ± .06	49.9 ± 4.5
7	"	28	2.0/14	1.68 ± .08	15.9 ± 1.3
4	"	18	4.0/ 9	1.44 ± .17	29.6 ± 4.6
7	KI	28	1.7/14	1.89 ± .06	121.6 ± 9.0
C. Effect of PTU with or without KI and thyroxine (diet 77A)					
7	PTU	28	0 / 0	8.27 ± .30	123.8 ± 17.6
7	PTU and thyroxine	28	2.0/14	3.35 ± .46	82.1 ± 3.5
7	PTU and KI	28	1.7/14	9.83 ±1.67	105.6 ± 14.4

* Solutions administered once per day.

† Mean and stand. error of mean. The latter calculated by the short method of Mantel(15):

$$\text{Stand. error (S.E.)} = \frac{\text{Range}}{\text{No. of animals}}.$$

the 5% level. This experiment was repeated with 8 Sprague-Dawley female rats weighing about 150 g. The mean T/S ratio obtained for 4 hypophysectomized rats was 11.0 ± 5.6, and for 4 thyroxine-treated hypophysectomized rats was 3.9 ± 0.6.

The results obtained, following administration of 2 µg thyroxine for 5, 8, and 14 days, and, in one experiment, of 4 µg for 9 days, are given in Table I B. Although there is a difference in iodine content between diet 77A and 277, as is indicated by the T/S ratio of the control group 137.2 vs 253, the extent of the depressing effect of thyroxine in the two groups is not significantly different.

The greatest depression of the T/S value was obtained by a thyroxine dose of 2 µg daily for 14 days (Table I B). A slightly greater depression occurred at a dose of 4 µg thyroxine daily for 9 days than at a dose of 2 µg for 8 days. The equivalent amount of iodine as KI for 14 days did not significantly depress the T/S value in this experiment, 121.6 ± 9 vs 137.2 ± 5.3 (Table I B).

Although the chronic administration of 0.025% propylthiouracil (PTU) resulted in a 10-fold increase in thyroid weight, there was no increase in the activity of the thyroid trap(7). Thyroxine caused a depression in trap activity and an inhibition of thyroid hypertrophy. The administration of KI, at a dose level equivalent to the iodine content of thyroxine, caused some depression in thyroid weight and a barely significant depression of T/S ratio (Table I C). The T/S ratios obtained from hypertrophied thyroid glands depressed with thyroxine are very similar to values obtained after hypertrophy and hypophysectomy(5).

Pretreatment of mice with 4 µg of thyroxine per day for 9 days was followed by the administration of a single dose of TSH, 40 hours prior to sacrifice. The mice receiving the TSH were divided into 2 groups, one receiving the equivalent of 0.5 mg and the other 1.5 mg USP thyrotrophin reference substance. Each group receiving the TSH was further subdivided into 2 groups, one of these receiving 1 mg PTU. The results are indicated in Table II. Thyroxine caused a pronounced depression of the thyroid iodide trap activity. When TSH and PTU were administered, the activity of the iodide trap was stimulated more than when only TSH was given.

TABLE II. Response of Thyroid Gland in Intact Mouse to Administration of TSH after Pretreatment with Thyroxine.

No. of mice	Dose of TSH (mg)*	Dose of PTU (mg)	T/S
4	0	0	29.6 ± 4.7†
4	.5	0	38.2 ± 4.7
5	.5	1.0	42.4 ± 2.5
4	1.5	0	53.9 ± 4.6
5	1.5	1.0	71.3 ± 9.1

* Equivalent to indicated quantity of USP thyrotrophin reference substance.

† Mean and stand. error of mean.

Discussion. Cortell and Rawson(11) and Shellabarger and Godwin(12) have reported that thyroxine exerts a depressant effect on the thyroid, independently of the effect it may exert through the pituitary. However, in neither case was the evidence direct. Halmi *et al.*(8) were the first to establish the directly depressant role of thyroxine on the thyroid gland when they demonstrated that thyroxine depressed the iodide trap to a greater extent than did hypophysectomy. This observation has now been confirmed, in both mice and rats, in a modified experiment in which hypophysectomized mice and rats received thyroxine, with a further depression in the T/S ratio resulting.

The activity of the mouse thyroid iodide trap is much greater than that of the rat (7,13), and either hypophysectomy or thyroxine are ineffective in completely depressing the iodide trap. The mouse iodide trap also appears to be more independent of hormonal control than that found in the rat. This condition does not appear to extend to other steps in the biosynthesis of the thyroid hormone(5). The level of hormonal independence was greatly increased by causing hypertrophy, prior to depression with thyroxine. This confirms similar results obtained after hypophysectomy(5).

The iodide trap of the thyroxine-depressed mouse thyroid gland responds to the administration of TSH(14), and this response is potentiated by the simultaneous administration of PTU(8). This mechanism in both mice and rats is not sufficiently sensitive to serve as an assay technic for thyrotrophin.

Summary. Chronic treatment with thyroxine was no more effective than hypophysectomy in depressing the thyroid iodide trap. TSH had some stimulating effect on the activity of the thyroid iodide trap. The combination of TSH and PTU caused a greater response, but insufficient to serve as an assay technic for thyrotrophin.

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The Saukett (Leon type) Strain of Poliomyelitis Virus in the Syrian Hamster. (21450)

Reginald L. Reagan, Sing Chen Chang, and A. L. Brueckner.

From the Live Stock Sanitary Service, Virus Laboratory, University of Maryland, College Park, Md.

The Saukett (Leon) strain of poliomyelitis virus has been passed serially in 16- to 18-day-old market Syrian hamsters by intracerebral injection. The Saukett strain virus and specific antiserum were supplied by Salk and Bennett, of the University of Pittsburgh. The pooled virus had been propagated in monkey kidney tissue cultures for 10 passages; the LD_{50} in rhesus monkeys by intracerebral injection was $10^{-6.5}$ to 10^{-7}. Serial passages in hamsters were effected by the intracerebral injection of 0.06 ml of tissue culture material (original) or the crude supernates of 20% ground brain suspensions in 0.85% NaCl solution. Infected hamsters showed symptoms of irritability and malaise, followed by paralysis 5 to 8 days after inoculation; animals so affected did not recover. Brains used for virus passage were taken from moribund animals. Virus of the tenth passage exhibited a titer of 10^{-4} in hamsters, and was neutralized fully by specific Saukett antiserum.

A dose of 0.06 ml of the Saukett tissue culture infected material was injected intracerebrally into each of 6 hamsters. These hamsters, as well as those of successive passages, appeared normal as long as 5 days after exposure to the virus. Symptoms of paralysis occurred in from 5 to 8 days in the exposed group. The hamsters from which brain material was taken for passage were sacrificed while moribund. A 20% brain suspension was prepared by grinding the brain tissue in a mortar with alundum and diluting with physiological saline. 0.06 cc of the brain suspension (after centrifugation at 2,000 rpm for 3 minutes) was passed serially by intracerebral inoculation through 10 passages; virus of the tenth passage exhibited a titer of 10^{-4} intracerebrally in Syrian hamsters, as Table I indicates. Neutralization tests were employed using brain suspensions from tenth intracerebral passage. The Saukett specific antiserum was also provided by the Poliomyelitis Research Center in Pittsburgh. These neutralization tests done intracerebrally in hamsters identified the virus as Saukett strain.

TABLE I. Hamster Passage of the Saukett Strain of Poliomyelitis Virus Intracerebrally in 10 Successive Passages.

No. animals inj. intracer.	No. animals paralyzed moribund or dead	No. days post-inoculation occurred
6	2	5, 8
6	2	5, 7
6	2	7, 8
6	4	5, 6, 7, 8
6	5	6, 6, 6, 6, 6
6	4	6, 6, 7, 7
6	5	5, 6, 7, 7, 7
6	6	5, 6, 7, 7, 7, 7
6	5	6, 7, 7, 7, 8
6	5	5, 7, 7, 7, 7

Summary. The Saukett strain of poliomyelitis virus was successfully carried serially through 10 Syrian hamster brain passages. The disease in hamsters was characterized by symptoms of irritability and malaise, followed by paralysis. Hamsters typically affected did not recover. These passages are being continued at the present time.

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Production of Streptococcal Infections in Pharynx of the Rabbit.* (21451)

STEPHEN I. MORSE, JAMES E. DARNELL, JR., AND ROBERT J. GLASER.
(Introduced by Carl G. Harford.) (With technical assistance of Barbara B. Pesch.)
From Immunology Division, Department of Internal Medicine, and the Oscar Johnson Institute for Medical Research, Washington University School of Medicine, St. Louis, Mo.

The existence of a relationship between antecedent group A streptococcal infections and rheumatic fever is widely accepted but the pathogenesis of rheumatic fever remains an enigma, at least in part because of the lack of a suitable experimental model in animals. It has been known for many years that skin infections in rabbits could be produced with hemolytic streptococci(1,2), and more recently this fact has been demonstrated to obtain with strains belonging to group A(3). Previous publications from this laboratory have described methods for the production of pneumonia in rats(4) and cervical lymphadenitis in mice with group A organisms(5). Because, however, in man streptococcal infections predominantly affect the pharynx, it was decided to attempt to devise a method of inciting pharyngeal infection in animals, with a view subsequently to assaying the effect of multiple infections on the heart and kidneys. Since there is evidence that rabbits can respond to certain antigenic stimuli with the development of non-bacterial inflammatory reactions characteristic of those due to hypersensitiveness(3,6), this animal was selected as the subject of the experiments herein described.

Methods. New Zealand rabbits, 6 to 10 weeks of age and weighing approximately 5 kilos were used. The animals were free from demonstrable respiratory infections. A strain of group A streptococci, identified as 1GL 23 (type 17), originally obtained from another laboratory in the past, was employed. After passage in mice, the organism was grown in beef infusion broth containing 10% sheep serum and 0.5% dextrose (BIB-SD), concentrated by centrifugation, and stored in small tubes at –72°C. For each experiment, the streptococci were grown in BIB-SD for 16 hours at 37°C, after which 0.5 ml was transferred to fresh medium and incubated for 4 hours. Ten-fold dilutions of the culture were made and blood agar plates poured with aliquots of the 10-6, 10-7 and 10-8 dilutions. The infecting inoculum contained on the average 7 x 10^6 organisms in broth. For induction of infection, each rabbit was given 0.06 g of sodium pentobarbital intraperitoneally as a basal anesthetic, after which ether was administered by mask until adequate relaxation was obtained. The jaws were held open widely by means of a self-retaining, spreading clamp and the tongue was pulled forward. For purposes of visualization, a metal tongue depressor equipped with a small cystoscope bulb as light source† was introduced and the posterior pharynx was exposed. Approximately 0.2 ml of the suspension of streptococci was injected into the peritonsillar region on either side through a #20 lumbar puncture needle attached to a 1.0 ml tuberculin syringe. In one series of experiments, rabbits were sacrificed at 4, 8, 16,

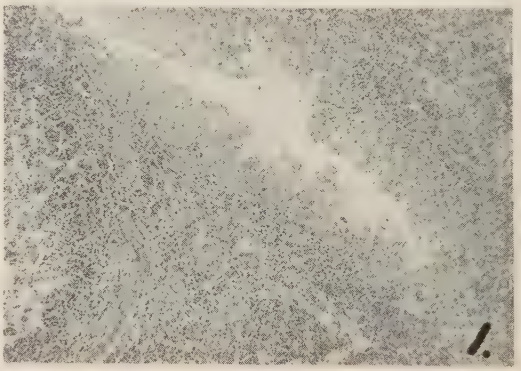

FIG. 1. Tonsillar section 48 hr after initiation of infection; follicular destruction and vascular engorgement as well as mucosal disruption are seen. Gram-Weigert stain. ×80.

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† Made by Mr. H. C. Lahay, Hamilton-Schmidt Surgical Instrument Co., St. Louis, Mo.

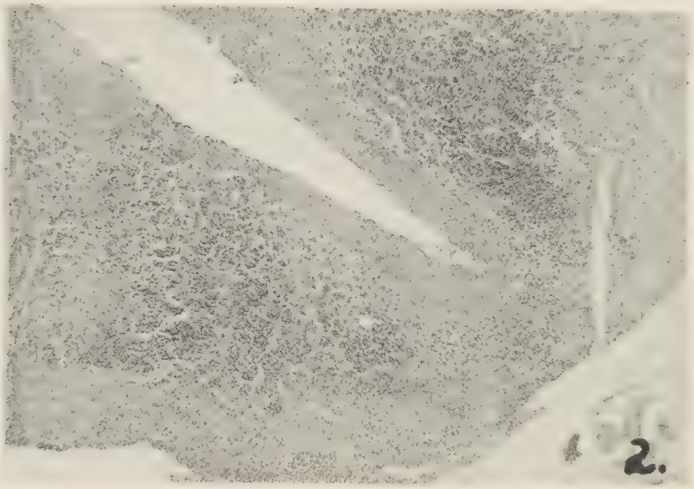

FIG. 2. Tonsillar section 48 hr after injection of sterile culture medium. Normal follicular architecture noted in the tonsillar lymphoid tissue with intact overlying mucosa. Gram-Weigert stain. ×80.

48 and 70 hours after induction of infection. Prior to sacrifice, blood for culture was removed aseptically from marginal ear veins periodically, beginning 15 minutes after inoculation. The total leucocyte counts were determined and rectal temperatures recorded at time the blood was cultured. The pharyngeal region was dissected free en bloc, fixed in 10% Zenker-formaldehyde solution, and cut sections were stained by one or more of three technics—Giemsa, Gram-Weigert and hematoxylin-eosin. The microscopic sections were cut in a coronal plane through blocks which contained both tonsils and the overlying tonsillar sulci. The naso-oro-pharyngeal structures were divided so that the bony roof of the oropharynx, 5 to 8 mm deep to the mucosa, was not included in the section. Other rabbits were subjected to infection in order to determine the anti-streptolysin O (ASO) response. Blood was obtained prior to inoculation and at weekly intervals thereafter, and the ASO titers determined by the method of Rantz and Randall(7).

Results. Gross Observations. Since the organisms were introduced beneath the epithelial surface, no obvious change was noted in the pharyngeal mucosa. In animals sacrificed at 48 and 70 hours, small abscesses were seen overlying the sheaths of the retropharyngeal muscles.

Incidence of Bacteremia. Fifteen minutes after inoculation, 6 of 10 animals had positive blood cultures. At one hour 3 cultures were positive. Subsequently, streptococci were recovered only rarely from cultures made 2, 3, 4, 8, 12 and 24 hours after infection was instituted. In no instance did a given animal have persistent bacteremia.

Temperature Response.‡ In general, a significant rise in rectal temperature(8) occurred 2 to 4 hours after induction of infection and persisted for as long as 48 hours; the elevations ranged from 1.5°C to 2.8°C (average 2.2°C). Injection of sterile broth in a normal control resulted in a single elevation of only 0.7°C at 30 hours.

Leucocyte Counts. Marked fluctuation in the total white blood cell counts was noted. No definite pattern could be ascertained, and there was no correlation between leucopenia and bacteremia.

Histologic Studies. Cellular inflammatory response was minimal but definite follicular destruction in the tonsil was evident and edema and vascular engorgement prominent (Fig. 1) when contrasted with control sections, obtained from animals injected with sterile BIB-SD (Fig. 2). Histologic changes were

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‡ Animals were not restrained during temperature determinations.

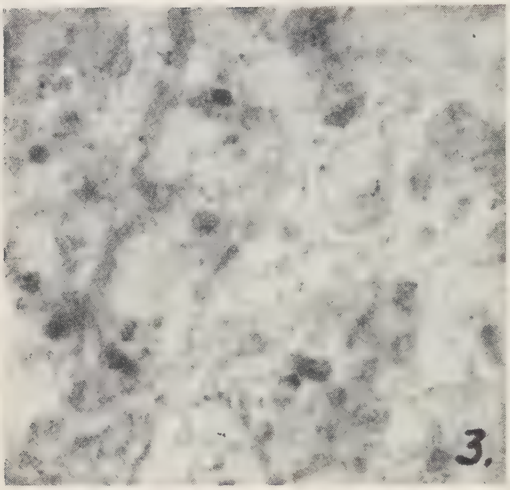

FIG. 3. Gram-positive cocci singly and in short chains in lymphoid tissue in the pharynx 48 hr after inoculation. Gram-Weigert stain. ×1000.

The photomicrographs were made by Mr. K. Cramer Lewis, Department of Illustration, Washington University School of Medicine.

of a more definite nature 18 to 24 hours after initiation of infection. Organisms were readily demonstrable in sections of the peritonsillar tissues stained by the Giemsa or Gram-Weigert technic (Fig. 3).

Anti-Streptolysin O Titers. The ASO responses of the seven animals subjected to infections and observed for the following 4 weeks are shown in Table I. Prior to infection the control titers were 0 or 12 units, and after infection the maximum level recorded was 333 units. A marked antibody rise occurred in four of the animals, and a minimal one in 3.

Discussion. Our study constitutes an additional approach to the study of experimental group A streptococcal infections, and one which simulates the conditions which obtain in man where involvement of the upper respiratory tract is the rule. The question of whether or not infection in this site is a prerequisite for the subsequent development of rheumatic fever cannot be answered with certainty. It is of interest, however, that some fragmentary evidence at least suggests this possibility(9). Employing the present method, studies are now under way to determine the effects of repeated infections on the cardiovascular system of rabbits(10).

TABLE I. Anti-Streptolysin O Response in Rabbits after Intra-pharyngeal Inoculation.

Animal No.	Prior to infection	Days after inoculation				
		7	11–12	14–18	21–23	27–30
33	0	12		12	50	0
37	0		50		12	12
38	12	12		100		250
39	0		50	12	12	
40	12	12		125	250	250
41	0	0		12	12	250
42	12		333		250	166

Intrapharyngeal inoculation is technically more difficult than either intranasal or intrabronchial inoculation, and the infection produced is less easily standardized and more difficult to study systematically. On the other hand, the following evidence indicates that infection was induced: (1) a consistent, significant rise in the animals' temperatures; (2) the presence of bacteria in the histologic sections and the development of small abscesses; and (3) significant rises in the ASO titers in a majority of the animals. In this regard, it should be emphasized that the transient bacteremia which occurred shortly after inoculation probably does not suffice to explain the antibody response since prolonged contact with streptococci is necessary, at least in humans, to incite significant rises in ASO(11).

Unfortunately the pharyngeal mucous membranes did not become involved; *i.e.*, true exudative pharyngitis was not seen. Attempts to induce such changes have been uniformly unsuccessful(12). Nonetheless, it is believed that the method described constitutes a useful adjunct for the study of experimental streptococcal infections and their effects.

Summary. 1. A method is described for production of Group A streptococcal pharyngeal infections in rabbits. 2. After intrapharyngeal inoculation, transient bacteremia usually occurred, followed by temperature elevations to 48 hours and significant rises in ASO titers 4 weeks later. 3. Bacteria were demonstrable in pharyngeal tissues at 48 hours, and in some instances small abscesses were seen. 4. The method described affords another means of studying effects of repeated group A infections under conditions simulating those which obtain in man.

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Effect of Trypsin and Chymotrypsin on the Granuloma Pouch.* (21452)

George Kleinfeld and David V. Habif.

From the Department of Surgery, Columbia University, College of Physicians and Surgeons, New York City.

Trypsin has an anti-inflammatory effect in clinical cellulitis and thrombophlebitis(1,2,3). We have also observed that the use of trypsin in the treatment of superficial thrombophlebitis usually produces prompt subsidence of inflammatory signs(4). Other workers report that trypsin inhibits the formation of egg-white edema in the rat(5). In view of the highly variable course of thrombophlebitis in the human, it was decided to investigate further the anti-inflammatory effect of trypsin using laboratory animals and a standardized, reproducible inflammatory lesion. The effect of chymotrypsin was also studied as it has greater fibrinolytic activity than trypsin and apparently is less toxic(6).

Materials and method. Male white rats of the Sherman strain, weighing between 150 and 250 g were used and the granuloma pouch as described by Selye(7) was used as the standard inflammatory lesion. To produce a granuloma pouch, an air sac is created on a rat's back by the subcutaneous injection of 25 ml of air. One ml of 1% croton oil (diluted with corn oil) is then injected into the lumen of the sac. The mildly irritating action of the croton oil causes the tissue lining the sac to proliferate into a thick wall of reparative tissue and the lumen of the sac becomes filled with a viscid, hemorrhagic exudate. The effect of trypsin and chymotrypsin on the formation of this tissue was studied by giving the enzymes in single daily injections beginning with the day of production of the pouch. The rats were divided into several groups, each group receiving the enzymes by intracardiac, intraperitoneal, or intramuscular injection. After 10-14 days, the rats were sacrificed and the pouches dissected free. The volume of the fluid contents of the pouch was measured and the pouch wall weighed. A control group with the inflammatory pouch was run with each experiment, the control animals receiving 0.9% saline in single daily injections.

Results. In several controlled experiments, it was noted that the growth of the pouches, as reflected by the volume of exudate and the weight of the pouch wall at the end of the experimental period, was not significantly affected by these enzymes in the dosages and routes of administration used. The results of a typical experiment are shown in Table I. It will be noted that the volume of pouch contents and the weight of the pouch wall are approximately the same for all groups. The difference in the means between the experimental groups and the control group is not statistically significant. Other experiments, using dosages of trypsin between 100 and 1000 units

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* Supported by a grant-in-aid from the Armour Laboratories, Chicago, Ill.

TABLE I. Effect of Trypsin and Chymotrypsin on Granuloma Pouch.

Group	No. of rats	Enzyme	Dose (units)	Route of inj.	Body wt (g) mean		Vol of exudate (ml) mean	Wt of pouch wall (g) mean & range
					Initial	Final		
1	7	Control	1 ml 0.9% saline	Intraper.	171	233	16	3.8 3.3–4.5
2	8	Trypsin	1000	Intramusc.	166	225	19	3.7 3.0–4.5
3	5	"	"	Intracard.	180	247	19	3.5 2.0–4.5
4	5	Chymotrypsin	"	Intracard.	168	198	11	2.7 1.7–3.5
5	6	"	"	Intraper.	170	220	27	4.8 3.9–5.7

per rat and chymotrypsin dosages between 1000 and 5000 units per rat, gave similar results.

An accessory control group with the inflammatory pouch was treated with cortisone (Cortone, Merck). These rats were given 25 mg/kg of cortisone in single, daily, intramuscular injections for 7 days after the production of the pouch. In every instance the formation of the pouch was strikingly inhibited and at the end of the experimental period the pouch wall was too thin to dissect free. The croton oil could be recovered from the subcutaneous tissues of the rats' backs without evidence of exudate formation. These findings are in accord with those of Selye(7) and they demonstrate the effect upon the pouch of a powerful anti-inflammatory agent.

Discussion. It is important to note that the reproducibility of the inflammatory lesion is a primary consideration in evaluating the effect of an anti-inflammatory agent. In this respect, the granuloma pouch is somewhat variable in its pattern of formation and ultimate size. However, the anti-inflammatory property of cortisone is strikingly demonstrated by its effect upon the pouch. On the other hand, trypsin and chymotrypsin, as administered in these experiments, produce no effect. We consider these results to be evidence indicating that these enzymes cannot alter the formation of granulation tissue, as can cortisone. However, we are reluctant to exclude the possibility of an anti-inflammatory effect of trypsin and chymotrypsin inasmuch as clinical and laboratory observations(1-4) do demonstrate such an effect upon thrombophlebitis and egg-white edema respectively. It may be that the characteristics of thrombophlebitis render it susceptible to the action of trypsin.

In some experiments, 1 ml of bacitracin-neomycin solution (1000 u/ml and 1% respectively) was injected into the pouch along with the croton oil to decrease the chance of infection. In these animals, the skin overlying the pouch became escharified and in many instances ulcerated, collapsing the pouch. Subsequent experiments were performed in which the development of pouches containing croton oil and bacitracin-neomycin solution, or either antibiotic alone (in the same concentration), were contrasted with control pouches containing 0.9% saline. It was found that the combination of croton oil and bacitracin-neomycin solution or bacitracin alone was sufficiently irritating to cause necrosis of the skin overlying the pouch. The combination of croton oil and neomycin did not produce this effect.

Summary. Trypsin and chymotrypsin, given parenterally through various injection routes, did not alter the growth of granulation tissue forming in response to a chronic sterile inflammation in the rat.

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Isolation of Desoxycorticosterone from Adrenal Venous Blood of the Dog; Effect of Hypophysectomy and ACTH.* (21453)

GORDON L. FARRELL, ELIZABETH W. RAUSCHKOLB, PAUL C. ROYCE† AND H. HIRSCHMANN. (Introduced by George Sayers)

From the Departments of Physiology and Medicine, Western Reserve University School of Medicine, Cleveland, O.

The isolation of 11-desoxycorticosterone from extracts of beef adrenals was first reported by Reichstein and von Euw(1). This finding was confirmed by Zaffaroni and Burton who analyzed beef adrenal extracts by paper chromatography(2). The influence of 11-desoxycorticosterone on electrolyte metabolism and its importance in the control of adrenocortical insufficiency are well known. Although Hechter and coworkers(3) described the presence of small amounts of 11-desoxycorticosterone in the perfusate from isolated beef adrenals, these workers expressed doubt that the steroid was actually a component of the adrenal secretion.

In the present study, a steroid which is in all probability identical with 11-desoxycorticosterone has been isolated from adrenal venous blood of the dog and demonstrated by arterial and adrenal venous concentration studies to be an adrenal secretory product. The secretion of this steroid has been shown to be under the control of the pituitary.

Isolation of 11-desoxycorticosterone from a pooled lot of extracts of adrenal venous blood. The non-polar components from chromatograms of a large pool of extracts of adrenal venous blood from normal dogs were examined for 11-desoxycorticosterone. The crude material corresponded to Fraction 14 described in an earlier publication(4). Upon chromatography in the system propylene glycol-hexane, 477 μg (all estimates of quantity are based on U. V. absorption assuming $\epsilon = 17{,}400$ (cf. 5)) of an apparently homogeneous fraction separated which migrated at a rate equal to that of 11-desoxycorticosterone. The substance exhibited maximum absorption at 239.5 mμ and reacted with phenylhydrazine to yield a product with a 340 mμ absorption maximum. The substance reduced ammoniacal silver nitrate. An aliquot mixed with an equal quantity of an authentic sample of 11-desoxycorticosterone chromatographed (propylene glycol-hexane, 40 hours) as a single band. When mixed with sulfuric acid (3 hours, room temperature) it exhibited maxima at 280, 360 and 420 mμ, characteristic of 11-desoxycorticosterone. The substance was bioassayed‡ for sodium-retaining activity at doses of 1, 4, and 16 μg, and found to possess sodium-retaining activity approximately equal to that of 11-desoxycorticosterone. The ratio of potency, isolated substance/11-desoxycorticosterone, was 0.76 ± 0.3. The infra-red absorption spectrum showed the characteristic maxima of 11-desoxycorticosterone but in addition several maxima attributed to impurities. These could be removed by acetylation and chromatography. To this end, the remainder of the isolated material (about 250 μg) was acetylated at room temperature (acetic anhydride:pyridine 1:2, 16 hours) and rechromatographed 8 hours in the system propylene glycol-hexane; a substance was found which migrated at the same rate as 11-desoxycorticosterone acetate (211 μg). An aliquot mixed with an equal quantity of 11-desoxycorticosterone acetate chromatographed as a single band (propylene glycol-hexane, 8 hours). The spectrum in sulfuric acid was identical with that of 11-desoxycorticosterone acetate. A sample of the acetate

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† National Science Foundation Predoctoral Fellow.

‡ Na and K excreted in a 5-hour period following steroid administration are analyzed by flame photometry. Each assay employs 48 5-day postadrenalectomy rats, distributed among 3 doses of the standard and 3 doses of the unknown substance. The response is expressed as the ratio Na/K.

TABLE I. 11-Desoxycorticosterone Secretion by Control (Sham-Operated) and Hypophysectomized Dogs.

Control					Hypophysectomized				
Group	No. dogs	ml blood	μg/L	μg/kg/hr $\times 10^2$	Group	No. dogs	ml blood	μg/L	μg/kg/hr $\times 10^2$
1	4	1661	24.3	17.1	1	5	1611	8.0	5.0
2	4	2435	37.9	22.8	2	2	730	4.3	2.0
3	4	1540	52.5	32.9	3	6	1946	15.7	8.0
4	3	1515	41.8	38.0					
	Mean		39.1	27.8				9.3	5.0
	S.D.*		11.7	9.4				5.8	3.0

* S.D. = Stand. dev. of pooled lots, from groups, about the overall mean.

was dissolved in carbon disulfide and examined in the infra-red. The spectrum included absorption maxima at 5.70, 5.78, 5.98 and 8.14 μ which indicate the presence of a 21-acetoxy-20-keto and a Δ^4-3-keto group(6). The absorption in the fingerprint region likewise was in good accord with that of authentic 11-desoxycorticosterone acetate. The remainder of the acetate was bioassayed for sodium-retaining activity at doses of 1 and 9 μg. The potency ratio, isolated substance/reference 11-desoxycorticosterone acetate, was 2.25 ± 1.3, which is not statistically different from unity. On the basis of these findings the isolated substance appears to be 11-desoxycorticosterone.

Demonstration of an adrenal venous-arterial concentration difference of 11-desoxycorticosterone. 1,110 Ml of arterial blood, and 1,250 ml of adrenal venous blood were collected over the same time interval from the femoral artery and lumboadrenal vein, respectively, of a large male dog. Blood pressure was maintained by transfusion of whole blood. The adrenal venous blood and the arterial blood were collected in vessels at 0°, extracted with chloroform promptly and processed and chromatographed(4) without delay. A quantity of 18.6 μg/L of a substance with the same mobility as 11-desoxycorticosterone was isolated from the adrenal venous blood. An aliquot of this material mixed with reference 11-desoxycorticosterone chromatographed as a single spot. The sulfuric acid chromogen was characteristic of 11-desoxycorticosterone. The arterial blood sample yielded no substance with selective absorption at 240 mμ in the 11-desoxycorticosterone region. The results of this experiment indicate that the isolated steroid is secreted by the adrenal.

Demonstration that the isolated steroid is not an artifact derived from the major steroid constituents of adrenal venous blood. Two mg of 17-hydroxycorticosterone and 1.7 mg of corticosterone were added to 500 ml of arterial blood. The mixture was chilled and extracted with chloroform by the same technic as routinely employed for extraction of adrenal venous blood. The extract was chromatographed in the usual manner(4). The region of the chromatograms which would have been expected to contain 11-desoxycorticosterone did not contain a measurable amount of α, β unsaturated ketone. This result is taken as evidence that the steroid isolated in this study was not derived by conversion from the major secretory products of the adrenal cortex during the course of its isolation.

Effect of hypophysectomy and ACTH on secretion of 11-desoxycorticosterone. The secretion of 11-desoxycorticosterone by the adrenal of dogs during the period 6 to 10 hours after hypophysectomy was compared to that of control sham-hypophysectomized dogs. Male mongrel dogs were maintained under light sodium pentobarbital anesthesia and hypophysectomized by the oral approach. Six hours after hypophysectomy the left lumboadrenal vein was cannulated for collection of adrenal venous blood. The adrenal vein and all collateral branches of the lumboadrenal vein were ligated. Volumes of 1,611, 730 and 1,946 ml of adrenal venous blood were collected from 3 groups of hypophysectomized dogs. Volumes of 1,661, 2,435, 1,540 and 1,515 ml were obtained from 4 sham-hypophysectomized control groups. An additional volume of 2,345 ml was collected from 4 hypophysectomized animals injected intravenously with 100 Units of ACTH§ during the collection period. The collected blood in each experiment was kept at 0° until time of extraction and analyzed promptly. The results are shown in Table I. An average of 39.1 μg/L (0.278 μg/kg body weight/hour) of 11-desoxycorticosterone was isolated from the adrenal venous blood of sham-hypophysectomized dogs. The adrenal venous blood from hypophysectomized dogs contained an average of 9.3 μg/L (0.050 μg/kg body weight/hour). Following the administration of ACTH to a group of hypophysectomized dogs the concentration was increased to 6.23 μg/L (0.602 μg/kg body weight/hour). It is evident from these data that the secretion of 11-desoxycorticosterone declines to a low level following hypophysectomy, and is stimulated by the injection of ACTH.

For purposes of comparison of relative concentrations in the adrenal effluent, 17-hydroxycorticosterone and corticosterone were also isolated and estimated. 17-hydroxycorticosterone was secreted at rates of 31.6, 2.4 and 17.4 μg/kg body weight/hour, and corticosterone at rates of 12.6, 1.6 and 11.1 μg/kg body weight/hour in the control, hypophysectomy and hypophysectomy-ACTH experiments, respectively.

Discussion. Lack of material has prevented us from making as complete a characterization of the isolated compound as would seem desirable. Our observations, nevertheless, strongly indicate that it is 11-desoxycorticosterone. They include 1) a demonstration of the functional groups, Δ^4-3-ketone and 21-hydroxy-20-ketone, by infra-red and ultraviolet spectroscopy as well as by other tests, 2) bioassay of the ability to induce sodium retention in adrenalectomized rats which equalled both for the free compound and its acetate that of the authentic reference specimens, 3) measurements of the chromatographic mobilities of the free steroid and its acetate which showed that these compounds could not be separated from the corresponding reference specimens in mixture chromatograms, 4) close agreement of the infra-red spectra of the isolated acetate and 11-desoxycorticosterone acetate and 5) close agreement of the spectra in sulfuric acid of the isolated compound and its acetate with those of the reference specimens. Since Casanova *et al.* (7) have reported that 9(11)-anhydrocorticosterone has an equal effect on the urinary Na/K ratio as 11-desoxycorticosterone and possesses similar chromatographic mobility, it should be noted that the isolated acetate cannot be identical with this dehydration product of corticosterone acetate since our material shows no absorption peak near 12.44 μ and gives no green fluorescence with sulfuric acid (7).

Comparison of the concentration of the steroid in arterial blood with that found in adrenal venous blood has demonstrated that the steroid is a secretory product of the adrenal cortex. The secretion of the steroid has been shown to decrease following hypophysectomy and to be stimulated by ACTH injection.

It has been well established that administered 11-desoxycorticosterone is a potent agent in sodium and potassium metabolism but it has remained obscure how important a role the steroid plays in the normal animal. In the dog the amount of 11-desoxycorticosterone secreted by the adrenal is small and its effect on electrolyte metabolism is only a small fraction of that exerted by the aldosterone which is present in the same volume of adrenal venous blood(8,9,10). Hechter(3) has observed that perfusion of 11-desoxycorticosterone through beef adrenals results in the formation of corticosterone. He suggests that 11-desoxycorticosterone may be present as a precursor in the formation of corticosterone; according to this view the small quantity of 11-desoxycorticosterone which is released from the adrenal is incidental to its role as an intermediate in the transformations which lead to the production of corticosterone.

The development of pathological changes in experimental animals treated with large doses of 11-desoxycorticosterone has led to the suggestion that selective increase of endogenous "mineralocorticoid" production may be of etiological significance in certain diseases in man. The findings reported here establish that 11-desoxycorticosterone is secreted in the dog at extremely low levels as compared to the major steroid components of adrenal venous blood. It is not to be expected, therefore, that 11-desoxycorticosterone makes a significant contribution to the metabolic activities of the adrenal cortex. However, the possibility of the occurrence in disease of a marked alteration in the steroid secretory pattern leading to an excessive secretion of 11-desoxycorticosterone cannot be entirely dismissed. Definitive statements as to the role of 11-desoxycorticosterone in the development of pathological states must be deferred until such time as more definitive experimental data are available.

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§ Wilson "Corticotropin Solution," 100 U in 200 ml 0.9% sodium chloride solution made to 0.01 N with NCl.

Summary. A biologically active compound has been isolated from adrenal venous blood of the dog. It is in all probability 11-desoxycorticosterone. Its concentration in adrenal venous blood has been shown to be markedly increased over that in arterial blood, indicating that it is secreted by the adrenal. The rate of secretion of the isolated substance has been found to decrease following acute hypophysectomy and to increase following intravenous ACTH injection.

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Inhibition by Cortisone of the Binding of New Histamine in Rat Tissues.
(21454)

RICHARD W. SCHAYER,* ROSA L. SMILEY, AND K. JANE DAVIS.

From the Rheumatic Fever Research Institute, Northwestern University Medical School, Chicago.

Two recent papers have presented data which has led their authors to speculate that cortisone interfered with the normal renewal of histamine in tissues which had been depleted by histamine-release agents. In the first report by Goth, *et al.*(1) Tween 20 was injected into dogs and a drop in blood pressure resulted, presumably from the released histamine. A second injection 24 to 48 hours later still produced a significant hypotensive effect. If however, the dogs were pretreated with cortisone the first injection of Tween 20 lowered the blood pressure, but the second did not. The authors postulated that cortisone had prevented the accumulation of the histamine liberated by the first injection of Tween. In the second report, Halpern, *et al.* (2) found that rat skin sensitivity to dextran, as measured by the Evans Blue spot formation test, was lost after repeated intradermal dextran injections. In normal rats the sensitivity to dextran was fully restored in 100 to 120 hours; in cortisone treated rats there was no restoration of sensitivity even after 120 hours.

The following experiments represent at-

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TABLE I. Effect of Cortisone on Binding of New Histamine in Rat Skin.

	Molecules of new histamine bound in 24 hr/g skin/10^6 molecules of injected L-histidine	
Controls	32, 33, 40, 47, 55, 65	Avg = 45
Cortisone-treated	7, 9, 10, 12, 14	Avg = 10

tempts to confirm these findings by measuring directly the effect of cortisone on the rate of binding of new histamine in rat tissues.

Experimental. A group of female rats, 100-110 g, were injected intramuscularly with 10 mg cortisone[†] per rat for 3 successive days. A similar group of controls received saline only. One hour after the final dose of cortisone each rat received intraperitoneally 1 mg C^{14} L-histidine (3.95 x 10^6 cpm/mg). After 24 additional hours the rats were killed, the shaved abdominal skin removed, weighed, and analyzed for C^{14} histamine by isotope dilution(3).

Results are shown in Table I. Cortisone produced a marked drop in the C^{14} histamine bound in the skin.

To confirm this finding and to test the possible effect on other organs, two rats were treated with cortisone as before, injected with 1.47 mg C^{14} L-histidine each and killed 48 hours later. Similar tissues from both rats were combined and analyzed. Results are shown in Table II; they are compared with values obtained using normal rats.

Discussion. Our results present direct evidence that pretreatment with cortisone produces a large decrease in the rate of binding of new histamine in rat abdominal skin. This effect also appears to be operative in the intestines but not in the stomach. In tissues other than skin, confirmation is necessary. Because of the large quantities of C^{14} L-histidine required for these experiments in intact animals further work on various tissues of rats and other species[‡] will be attempted using *in vitro* technics. By these technics we have already amply confirmed the effect of cortisone on skin histamine.

TABLE II. Effect of Cortisone on Binding of New Histamine in Rat Tissues.

	Molecules of new histamine bound in 48 hr/g tissue/10^6 molecules of inj. L-histidine	
	Normal	Cortisone-treated[†]
Abdominal skin	111[*]	11
Intestines (large plus small)	9, 11, 12, 20	4
Stomach	31, 34, 41, 98	50

[*] This value is avg of 8 determinations (ranging from 72 to 187) obtained by experimental procedures similar to those used here. These experiments have been published(5).

[†] Values obtained from analysis of combined tissues of 2 rats.

The series of events through which L-histidine is transformed to bound histamine may be complex. The mode of interference by cortisone may be through inhibition of histidine decarboxylase, alteration of the binding mechanism, changing permeability so that histidine does not reach the site of decarboxylation and binding, or other causes. This remains to be clarified. Our experiments do not offer evidence either for or against Halpern's concepts of "combined" and "labile" forms of bound histamine.

It seems likely that the observations of Goth and Halpern were based on this same phenomenon; however the return of sensitivity they observed may have been due to replenishment of only a small fraction of the histamine originally released. Using the data of Table I, and calculating according to our previous method(4) it can be shown that roughly 2 μg of new histamine are added per gram of rat abdominal skin per day; the total histamine content of rat abdominal skin is about 30 μg per gram. Furthermore, the rate of binding of new histamine is not significantly greater in depleted rats than in normal ones(5). It seems likely, therefore, that the mechanism of replenishment of bound histamine in tissues involves not an increase in the rate of binding of new histamine, but a decrease in the rate of slow, spontaneous release of bound histamine which is probably proportional to the total number of molecules of histamine present in bound form. It would be expected that restoration of equilibrium (normal histamine concentration in the skin) would require a very long period of time; this has been directly observed by Feldberg and Talesnik(6).

[†] Cortone, Merck.

[‡] Species differences may be very important; we previously found that in mice cortisone treatment produced no marked abnormality in the amount of C^{14} histamine bound by the entire animal following administration of C^{14} L-histidine(7).

Summary. Injected cortisone strongly inhibits the rate of binding of new histamine in rat skin.

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Cortisone-Like Effect of Hematophorphyrin and Sunlight on Anaphylaxis in Mice.[*] (21455)

FRANK H. J. FIGGE AND GEORGE C. PECK.

From the Department of Anatomy, University of Maryland Medical School, Baltimore, Md.

During studies on the effect of hematoporphyrin on hypophyseal-adrenal relationships (1), it was found that small doses (0.6-2.0 mg) of hematoporphyrin did not protect horse serum-sensitized mice from a shocking dose of horse serum. However, when the porphyrin exceeded a total dose of 4 mg per mouse, the hematoporphyrin did exert a protective influence, in that a significantly higher percentage of porphyrin-treated mice survived a shocking dose of horse serum. In spite of these relatively clear-cut results, it appeared desirable to investigate the possible factors responsible for the occasional inconsistencies observed. Since hematoporphyrin is a light-sensitizing substance, it was conceivable that some of the variability in different experiments could have been due to non-controlled exposure of the animals to light. The present experiments were designed to test this hypothesis.

The results indicate that when a 2 mg dose of hematoporphyrin was followed by short controlled exposures of the mice to sunlight, they were definitely protected from the lethality of anaphylactic reactions. The degree of protection varied directly with the length of exposure to sunlight. A fifteen minute exposure of hematoporphyrin-treated mice protected them from lethal anaphylactic shock to the same degree as cortisone(1,2).

Methods and materials. Three hundred and fifty-eight mice[†] were sensitized to horse serum by injecting 1 cc of neutral undiluted horse serum intraperitoneally every second day until a total of 4 cc had been administered to each mouse(3). Following a 4 week sensitization period, the mice were then divided into 2 groups, *viz.*, no hematoporphyrin, and hematoporphyrin-treated (Table I). Two hundred and sixteen mice were not injected with hematoporphyrin. Of these, 184 received no additional treatment before the shocking dose of horse serum was given. Thirty-two served as sunlight controls and were subdivided into 3 groups which received 3, 10, and 15 minute exposures to sunlight before receiving the shocking dose of horse serum. In the hematoporphyrin-treated group, 142 mice were injected intraperitoneally with either 0.6 or 2

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[†] Mice in this experiment were the Strong A Strain or LCS_a Strain (derived from crossing a CBAN with Strong A). Mice of these strains have been inbred for 90 and 26 generations respectively, and are thus quite uniform.

TABLE I. Survivorship Rates. Influence of hematoporphyrin and sunlight on lethality of anaphylaxis in horse serum-sensitized mice.

Postsensitization treatment	No. of mice	No. survived	% survived ± S.E.
Control (none)	184	49	27 ± 3
Sunlight control			
Sunlight— 3 min.	11	2	18 ± 12
10	11	1	9 ± 9
15	10	3	30 ± 15
Total sunlight control	32	6	19 ± 7
Hematoporphyrin			
Injected with 0.6 mg	35	2	6 ± 4
2.0	19	2	11 ± 7
Total hematoporphyrin	54	4	8 ± 4
Hematoporphyrin (2 mg) + sunlight			
3 min. exposure	30	11	37 ± 9
10	32	24	75 ± 8
15	26	24	92 ± 5
Total hematoporphyrin & sunlight	88	59	67 ± 5

mg of hematoporphyrin in 2 doses. One half of the dose of hematoporphyrin was given 72 hours; the other half was given 48 hours before the shocking dose of horse serum. Eighty-eight of the mice that received 2 mg of hematoporphyrin were subsequently subjected to 3, 10, or 15 minute exposures to direct sunlight 24 hours before the shocking dose of horse serum was injected. Anaphylactic shock reactions were induced by rapidly injecting 1 cc of undiluted horse serum into the tail vein.

Results. Survivorship rates of the several treatment groups as well as the number of animals, are given in Table I.

Statistical analysis[‡] was designed to answer the following questions: 1. Did sunlight *per se* affect survivorship? 2. What was the effect of small doses of hematoporphyrin upon survivorship in the absence of exposure to sunlight? 3. Did exposure to sunlight affect the survivorship of animals treated with hematoporphyrin? And, specifically, did duration of exposure of porphyrin-treated mice to sunlight act as a determinant of survivorship? To facilitate such analysis, the data in Table I were rearranged as shown in Table II.

The rates shown in Cells IV and VI are based upon the combined experience of the animal groups exposed for 3, 10, and 15 minutes respectively at the zero and 2 mg levels of hematoporphyrin. In assessing the variation found between treatment groups the t-test has been employed to determine whether observed differences are statistically significant.

The data in Cells IV and I provide the relevant information to test whether sunlight, in the absence of any other experimental variable, affects survivorship. The observed difference between the 32 mice exposed to sunlight alone and the 184 controls is not significant ($t = .96$; $p > .05$). It would appear then that sunlight by itself does not affect survivorship. Comparison of Cell I with Cells II and III combined constitutes a test of the main effect of low doses of porphyrin upon survivorship after a shocking dose of horse serum. Among the 54 animals injected with porphyrin following sensitization, only 4 mice or 8% survived, as compared with 27% for control sensitized animals, not treated with hematoporphyrin. This difference is statistically significant ($t = 2.9$; $p < .01$).

In a previous study(1) some evidence of protection from lethal anaphylactic shock was found with dosage levels above 4 mg of porphyrin. Within the dosage interval used in these experiments (.6-2 mg) the protective influence was not observed. On the contrary, the post-sensitization treatment of mice with .6-2 mg of hematoporphyrin had a definitely adverse influence on the survivorship rate.

The analysis thus indicates that neither sunlight nor porphyrin in doses less than 2 mg when employed separately increase survivor-

TABLE II. Survivorship Rates According to Exposure to Direct Sunlight and Dosage Level of Hematoporphyrin.

Hematoporphyrin (mg)	Exposure to direct sunlight No	Yes
0	I $\frac{49}{184} = 27\%$	IV $\frac{6}{32} = 19\%$
.6	II $\frac{2}{35} = 6\%$	V —
2.0	III $\frac{2}{19} = 11\%$	VI $\frac{59}{88} = 67\%$

[‡] We are indebted to Matthew L. Taback for advice on statistical treatment of these data.

TABLE III. Survivorship Rates According to Duration of Exposure to Sunlight and Treatment with Hematoporphyrin.

Porphyrin treatment	Duration of exposure to sunlight (min.) 0	3	10	15
0	27	18	9	30
2 mg	11	37	75	92

ship following shocking doses of horse serum. The low dosage (0.6-2 mg) of porphyrin, on the contrary, significantly reduces survivorship.

The combined effect of sunlight and porphyrin was initially evaluated by comparing cells VI and I. The survivorship rate among animals treated with porphyrin and exposed to sunlight was 67% as compared with 27% in the control animals, (no sunlight, no porphyrin). This is a statistically significant difference.

The protective effect of duration of exposure upon porphyrin-treated mice is expressed quantitatively in Table III.

In the presence of hematoporphyrin, survivorship was directly related to the duration of exposure to sunlight. This constitutes a most unusual effect when it is recalled that hematoporphyrin in doses less than 2 mg, when employed by itself acts as a toxic agent, and sunlight alone has no demonstrable effect.

The protection from lethal anaphylactic shock of 2 mg of hematoporphyrin and 15 minutes of sunlight is almost as great (92% survivorship) as with cortisone(1,2) which gave full protection (100%).

Discussion. It was previously shown that in contrast to cortisone, ACTH did not protect mice from lethal anaphylactic shock. There is considerable disagreement in the literature concerning the effect and mode of action of ACTH and cortisone in experimental hypersensitive states(4-15). In addition to the results reported in the preceding paper(1), there are numerous instances in which ACTH and cortisone do not elicit identical responses. A more specific appraisal of these two hormones and their actions would be desirable. It is thus of interest that mild light shock (hematoporphyrin plus 15 minutes in sunlight) has an effect similar to cortisone in protecting animals from lethal effects of anaphylactic shock. The mechanism by which hematoporphyrin and sunlight acts to inhibit lethal effects of anaphylaxis is entirely obscure, but may ultimately lead to our understanding of how cortisone accomplishes the same end results. It had been postulated that cortisone inhibited antibody formation(4,5,8), but this hypothesis did not fit the observations of Mirick(11). In some human subjects, the latter investigator found that ACTH did not suppress antibody formation, but in some cases did suppress anaphylactic reactions despite abundant circulating antibody. Others(2,11, 15) have also presented evidence to show that the protective influence of ACTH and cortisone was not related to the suppression of antibodies. Some investigators have suggested that ACTH or cortisone may interfere with the reaction of the antigen and antibody in the host(11), but Wedgwood(15) has presented evidence to show that ACTH did not inhibit development of antibody and in addition, did not inhibit interaction of antibody and antigen, for they were able to demonstrate a decrease in the titre of serum complement after a challenging dose of antigen in ACTH-treated animals. They concluded that ACTH in some way alters the capacity of the tissues to react to the usually irritant antigen-antibody combination. This hypothesis was also alluded to by Mirick(11) who admitted that while ACTH or cortisone might interfere with the reaction of antigen and antibody in the host, that their influence was most likely related to an interference at some subsequent point in the chain of reactions involved.

In the experiments reported here, we can be certain that neither the porphyrin nor the sunlight had any influence on rates of antibody production, as they were administered after the antibodies had been produced. It is possible that the injection of porphyrin and exposure to sunlight gives rise to the formation or liberation of substances capable of competing with or interfering with the union of antibody with antigen. At the present state of our knowledge, further speculation is not justified. It would seem advisable to initiate experiments designed to study and compare the action of porphyrin and sunlight with the effects of cortisone.

Summary. Forty-nine of 184 control horse serum-sensitized mice (27%) survived a shocking dose of horse serum. Three to 15 minutes exposure of sensitized mice to sunlight has no influence on survivorship in these mice. Low doses (0.6 - 2 mg) of hematoporphyrin significantly increased the lethal effects of anaphylaxis in 54 horse serum-sensitized mice since only 8% survived. A small dose of hematoporphyrin (2 mg) and 3 - 15 minutes exposure to sunlight decreased lethality of shocking doses of horse serum administered to sensitized mice. The protection afforded was directly related to the length of exposure. Twenty-four of 26 sensitized mice (92%) injected with 2 mg of hematoporphyrin and subsequently exposed for 15 minutes to direct sunlight survived a shocking dose of antigen. This is an effect similar to that exerted by cortisone and of the same order of magnitude.

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Hydrodynamic Model of A 3-Compartment Catenary System with Exchanging End Compartments. (21456)

ALBERT A. PLENTL AND MARY JANE GRAY.

From the Department of Obstetrics and Gynecology, Columbia University, College of Physicians and Surgeons and the Presbyterian Hospital, New York City.

For the study of fluid exchange mechanisms, the maternal organism can be arbitrarily divided into 3 major compartments: the total maternal body water, the fetal body water and the amniotic fluid. These compartments exchange water and electrolytes at specific rates, and they constitute a unique system in which all compartments exchange with each other. In order to investigate the mechanism of this exchange, all three compartments should be available for study, but one of them, the fetus, will probably remain inaccessible during the course of *in vivo* experiments. Zilversmit and Shore(1) proposed the use of hydrodynamic models for the study of complex metabolic processes, and Sheppard and Householder(2) suggested electrical analogies for similar problems. Such models might supply useful information on the behavior of the inaccessible compartment, and might also provide experimental evidence for the correctness of the theoretic treatment.

It is the purpose of the present experiments to determine with hydrodynamic models how transfer rates can be calculated from "time-activity" curves under a set of conditions

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analogous to the particular biologic system. To this end it is necessary (1) to outline the mathematical basis, and (2) to apply this treatment to experimental data likely to be encountered in clinical or biological experiments.

Theoretic considerations. A schematic presentation of the system is given in Fig. 1. Compartments 1, 2 and 3 exchange their species with each other at 6 rates, which may assume any value, except for the restriction that the sum of transfer rates *in* and *out* of each compartment must be equal. This implies that the conditions of a steady state (uniform mixing of tracer and constant volume) are met. Let $x =$ concentration of indicator (or specific activity of tracer) in compartment 2 (amniotic fluid); $y =$ concentration of indicator in compartment 1 (mother); $z =$ concentration of indicator in compartment 3 (fetus); $S_x =$ size of compartment 1; $S_y =$ size of compartment 2; $S_z =$ the size of compartment 3.

If ρ_1----ρ_6 represent transfer rates of tracer in ml/hr in directions defined in Fig. 1, the change in x, y and z with respect to time will be:

$$\frac{dx}{dt} = -\frac{\rho_4 + \rho_5}{S_x}x + \frac{\rho_6}{S_x}y + \frac{\rho_3}{S_x}z$$

$$\frac{dy}{dt} = \frac{\rho_5}{S_y}x - \frac{\rho_2 + \rho_6}{S_y}y + \frac{\rho_1}{S_y}z$$

$$\frac{dz}{dt} = \frac{\rho_4}{S_z}x + \frac{\rho_2}{S_z}y - \frac{\rho_1 + \rho_3}{S_z}z$$

This is a linear differential equation with constant coefficients which, for convenience, can be written as:

$$\begin{aligned} &\frac{dx}{dt} + ax + by + cz = 0 \\ &\frac{dy}{dt} + a_1x + b_1y + c_1z = 0 \quad \text{........} \quad (1) \\ &\frac{dz}{dt} + a_2x + b_2y + c_2z = 0 \end{aligned}$$

where the constants a, b, c, a_1, b_1, c_1, a_2, b_2, and c_2 correspond to terms $\rho_4 + \rho_5/S_x$, $-\rho_6/S_x$, $-\rho_3/S_x$ etc. For the solution of this equation it is assumed that x, y and z can be expressed by the integrals:

$$x = \alpha e^{rt};\ y = \beta e^{rt};\ z = \gamma e^{rt} \quad \text{......} \quad (2)$$

Differentiation of (2) and substitution in (1) followed by elimination of the term e^{rt} then gives:

$$\begin{aligned} &(a + r)\alpha + b\beta + \gamma c = 0 \\ &a_1\alpha + (b_1 + r)\beta + \gamma c_1 = 0 \quad (3) \\ &a_2\alpha + b_2\beta + \gamma(c_2 + r) = 0 \end{aligned}$$

These relations require that the determinant $F(r) = 0$

$$F(r) = \begin{vmatrix} (a + r) & b & c \\ a_1 & (b_1 + r) & c_1 \\ a_2 & b_2 & (c_2 + r) \end{vmatrix} = 0 \quad \text{........} \quad (4)$$

The constant *r* has, therefore, three roots one of which is zero. Since equation (3) holds for all values of *r*, each of these has a corresponding value for α, β and γ and the specific solution of the equation becomes:

$$\begin{aligned} x &= \alpha_1e^{r_1t} + \alpha_2e^{r_2t} + \alpha_3e^{r_3t} \\ y &= \beta_1e^{r_1t} + \beta_2e^{r_2t} + \beta_3e^{r_3t} \quad \text{......} \quad (5) \\ z &= \gamma_1e^{r_1t} + \gamma_2e^{r_2t} + \gamma_3e^{r_3t} \end{aligned}$$

since $r_3 = 0$, the term e^{r_3t} is equal to unity.

Thus,

$$\begin{aligned} x &= \alpha_1e^{r_1t} + \alpha_2e^{r_2t} + \alpha_3 \\ y &= \beta_1e^{r_1t} + \beta_2e^{r_2t} + \beta_3 \quad \text{........} \quad (6) \\ z &= \gamma_1e^{r_1t} + \gamma_2e^{r_2t} + \gamma_3 \end{aligned}$$

and the general solution under these conditions is

$$\begin{aligned} X &= C_1\alpha_1e^{r_1t} + C_2\alpha_2e^{r_2t} + C_3\alpha_3 \\ Y &= C_1\beta_1e^{r_1t} + C_2\beta_2e^{r_2t} + C_3\beta_3 \quad \text{..} \quad (7) \\ Z &= C_1\gamma_1e^{r_1t} + C_2\gamma_2e^{r_2t} + C_3\gamma_3 \end{aligned}$$

where C_1, C_2 and C_3 are arbitrary constants.

The experimental data on the contemplated biologic experiments will be given in terms of curves showing the variation of x, y and z as a function of time. In other words, the experimental data will give the specific or general solution from which the original differential equation will have to be calculated. It is possible to determine the values of the three constants for each α, β and γ as well as the exponential constants r_1 and r_2 by a graphic solution of the data and thus construct 9 equations from which the 9 constants a, b, c, a_1, b_1, c_1, a_2, b_2 and c_2 can be calculated.

Substitution in equation (3) gives:

$$\begin{aligned} (a + r_1)\,\alpha_1 + b\beta_1 + c\gamma_1 &= 0 \\ (a + r_2)\,\alpha_2 + b\beta_2 + c\gamma_2 &= 0 \\ (a + r_3)\,\alpha_3 + b\beta_3 + c\gamma_3 &= 0 \quad \ldots\ (8) \\ a_1\alpha_1 + (b_1 + r_1)\,\beta_1 + c_1\gamma_1 &= 0 \\ &\vdots \\ &\text{etc.} \end{aligned}$$

Because of the "steady state" conditions and the fact that the total amount of indicator remains within the three compartments, the sum of x, y and z is constant during the period of the experiment, hence (from equation (6)) $\alpha_3 + \beta_3 + \gamma_3 =$ constant, and (from equation (1))

$$\begin{aligned} a + b + c &= 0 \\ a_1 + b_1 + c_1 &= 0 \quad \ldots\ldots\ldots\ldots\ (9) \\ a_2 + b_2 + c_2 &= 0 \end{aligned}$$

The last set of equations can also be derived from the fact that the volume or total amount of exchangeable species is constant for each compartment which requires that the total amount leaving each compartment must be equal to the amount entering it, hence

$$\rho_1 + \rho_3 = \rho_2 + \rho_4$$

and $\ldots\ldots\ldots\ldots\ (10)$

$$\frac{\rho_1 + \rho_3}{S_x} - \frac{\rho_2}{S_x} - \frac{\rho_4}{S_x} = 0$$

or $\quad a + b + c = 0$

Substitution of the values obtained by graphic solution of the experimental curves in equations (8) then gives the solution for the coefficients of equation (1). For example, the value of a_2 can be expressed in terms of α_1, α_2, β_1, β_2, γ_1, γ_2, r_1 and r_2:

$$a_2 = \frac{r_1 \dfrac{\gamma_1}{\gamma_1 - \beta_2} - r_2 \dfrac{\gamma_2}{\gamma_2 - \beta_2}}{\dfrac{\alpha_2 - \beta_2}{\gamma_2 - \beta_2} - \dfrac{\alpha_1 - \beta_1}{\gamma_1 - \beta_1}} = -\frac{\rho_4}{S_z}$$

The calculation of the transfer constants ρ_1 to ρ_6 from these coefficients requires that the volumes (or size) of the compartments are known.

For the immediate experimental proof the following approaches can be used: (1) the exchange rates ρ_1 to ρ_6 can be set at desired values thus determining the coefficients of the original differential equation; the experimental curves can then be compared with the theoretic solutions, (2) Graphic interpretation of the experimental curves can be used to calculate the values of ρ_1 to ρ_6 from the values of r_1, r_2, α_1, α_2, β_1, β_2, γ_1 and γ_2. The latter approach has been decided upon in order to serve as an example for the interpretation of a number of biologic experiments now in progress.

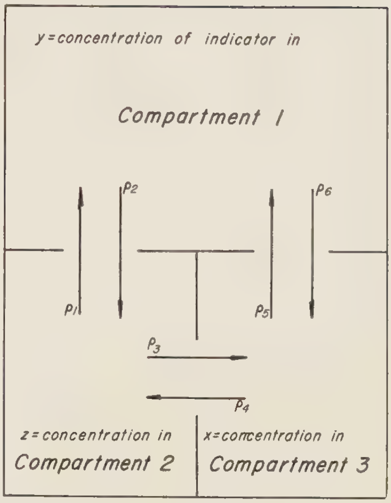

FIG. 1. Schematic presentation of the 3-compartment system defining the 6 exchange rates.

Experimental. The 3 hypothetical compartments of the apparatus, fetus, amniotic fluid and mother, are represented by glass tanks containing 1500, 1500 and 29000 cc of 0.01 M phosphate buffer. Each compartment was provided with a suitable mechanical stirrer to insure adequate mixing of the indicator. The transfer between these compartments was accomplished by means of 6 mechanical pumps adjusted to deliver specific volumes per unit time. These rates were chosen to fall within the expected range of the biologic system, each pump representing a transfer rate. With reference to Fig. 1 they were set to deliver $\rho_1 = 2040$, $\rho_2 = 1930$, $\rho_3 = 210$, $\rho_4 = 320$, $\rho_5 = 110$, and $\rho_6 = 220$ cc/hr.

In 3 successive experiments, the indicator (Evans blue T-1824) was first added to compartment 1, then to compartment 2 and finally to compartment 3. The change in concentra-

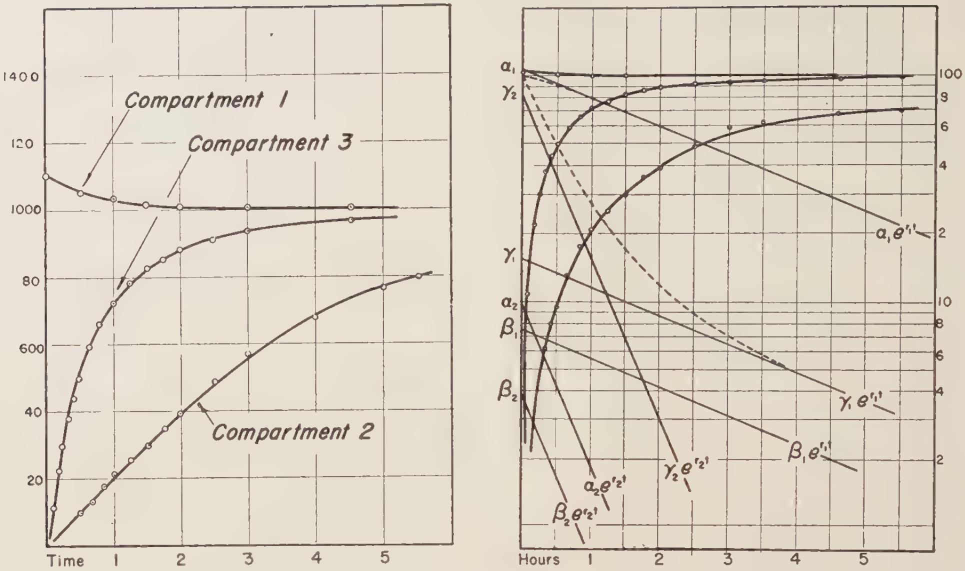

FIG. 2. Graphic presentation of data obtained in Exp. 1; compartment 1 originally contained all the indicator.

tion of the indicator for each compartment was determined colorimetrically, and the results were plotted as a function of time (Fig. 2, 3, and 4) in Cartesian and semi-log coordinate systems. The 3 exponential terms for each of the three dependent variables x, y and z were obtained by graphic solution of the semi-log plots. The values of the exponential constants r_1 and r_2 for the three experiments were found to be nearly identical within the experimental error of the method. The values of the constants α_1, β_1, and γ_1 were estimated from the points of intersection of the lines $\alpha_1 e^{r_1 t}$, $\beta_1 e^{r_1 t}$ and $\gamma_1 e^{r_1 t}$ at zero time. α_2, β_2 and γ_2 were calculated from the preceding values according to equation 5 by setting t,

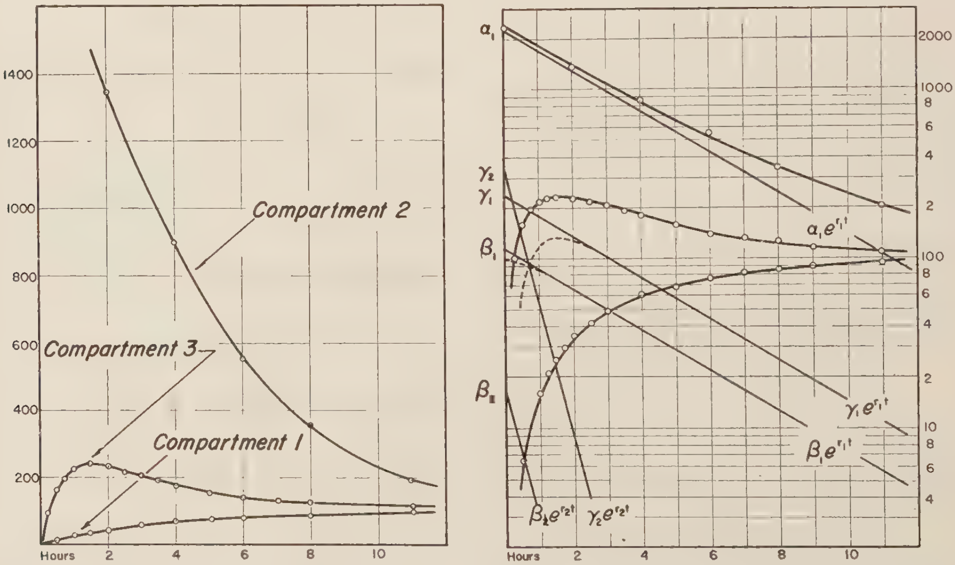

FIG. 3. Graphic presentation of data obtained in Exp. 2; compartment 2 originally contained all the indicator.

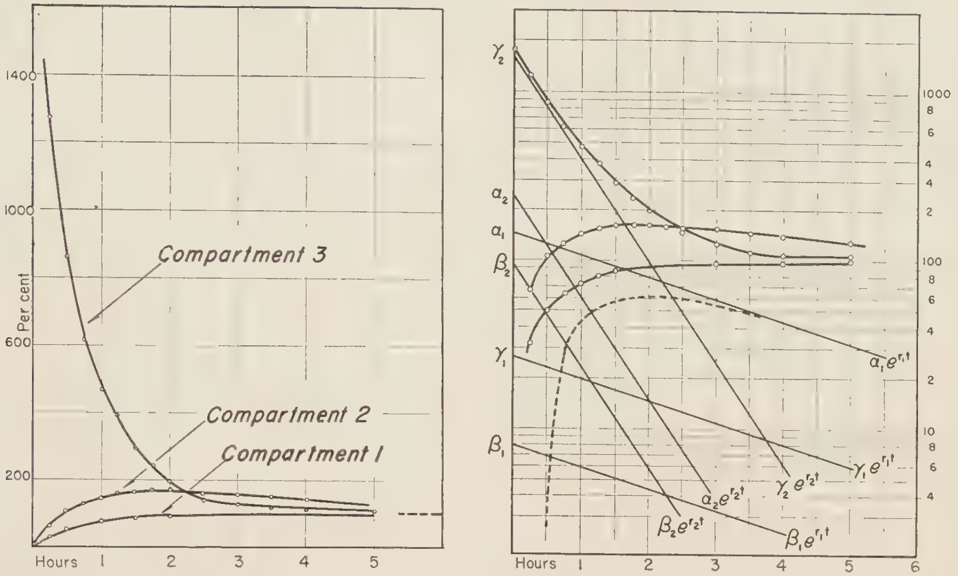

FIG. 4. Graphic presentation of data obtained in Exp. 3; compartment 3 originally contained all the indicator.

y and z equal to zero. The values for the 6 specific rate constants were then calculated in a manner similar to that illustrated in equation 11. In Table I, the theoretical values are compared with those calculated from experimental data.

The theory demands that the differential equation (1) has a specific and general solution. The specific solution (equation 6) for each of the three experiments is determined by the values of the constants α, β etc., and the general solution (equation 7) differs from it by the arbitrary constants C_1, C_2 and C_3. In each of the 3 experiments a different compartment initially contained all the indicator, while the 6 rate constants remained the same. The exponential constants r_1, r_2 and r_3 must therefore be the same for the 3 experiments, but the concentration curves and the values for the constants α, β etc. are different for each.

TABLE I. A comparison of the pre-determined transfer rates with the values calculated from graphic solution of "Time-Activity" Curves. The rates have the dimension of cc/hr, and their direction is defined in Fig. 1. In each of the 3 experiments another compartment initially contained all the indicator.

	Theory	Found		
		Exp. I	Exp. II	Exp. III
ρ_1	2040	2074	1920	1834
ρ_2	1930	1820	1850	1927
ρ_3	210	229	—	215
ρ_4	320	362	360	346
ρ_5	110	116	100	134
ρ_6	220	260	—	253

The fact that the majority of the constants are interrelated may be of considerable practical significance because some of them cannot be determined with sufficient accuracy due to extreme differences in size of the compartments and transfer rates. The simultaneous or successive use of two tracers could enable the investigator to carry out a pair of experiments simultaneously or in brief succession, and choose the most reliable experimental data for the calculation of the coefficients.

In the specific, though trivial application of these equations to the physiology of the amniotic fluid where transfer rates and volumes of the compartments differ widely, it can be demonstrated that the majority, but not all, of the transfer rates can be calculated with an acceptable degree of accuracy; the individual transfer rates and their sum *out* of the compartment initially containing all the indicator (or tracer) can be determined quite accurately, but the calculated individual transfer rates in the opposite direction will be unreliable, despite the fact that their sum is known. Thus, in the third experiment (compartment 3 initially containing all the indicator), the transfer rates ρ_4 and ρ_5 can be estimated, while only the sum of ρ_3 and ρ_6 is known. Placing the tracer into compartment 2 (Experiment 2), would permit an accurate determination of ρ_2 and ρ_3. In similar manner, ρ_1 and ρ_6 can be determined from the data of Exp. 1. Considerable difficulty should be anticipated in biologic studies analogous to Exp. 1 and 2, partly because of inadequate initial mixing of the tracer in the larger compartment (maternal body water), and partly because of the inaccessibility of the second compartment (fetus).

Summary. A hydrodynamic model of a 3 compartment system analogous to the maternal organism has been constructed. Its schematic representation is that of a catenary 3 compartment system in which the end compartments exchange. The mathematical basis for this system has been reviewed and experimental "time-activity" curves for the 3 compartments used to calculate the transfer rates.

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Peroxide Production and Peroxidase Activity of Sulfathiazole Sensitive and Resistant Strain of *Streptococcus mitis*.* (21457)

WILLIAM E. CLAPPER, GRACE H. MEADE, AND GABRIEL HAIBY.†

From the Lovelace Foundation for Medical Education and Research, Albuquerque, and Department of Microbiology, University of Colorado Medical School, Denver.

Determinations of hydrogen peroxide made at 24-hour intervals showed appreciable amounts in aerated broth cultures of several strains of *Streptococcus mitis*, but none in cultures of trained sulfathiazole resistant strains(1). A similar observation was made by MacLeod(2) with pneumococci. The cause of this apparent loss of peroxide producing ability interested us since neither catalase nor cytochrome oxidase could be shown to be present in the resistant cells. Seeley and VanDemark(3) have reported that a strain of *Streptococcus faecalis* grown aerobically produced an adaptive enzyme which destroyed added hydrogen peroxide. Since our resistant strains have many of the properties of *S. faecalis*(4), a similar peroxidase was looked for in the resistant strains. A quantitative comparison of the hydrogen peroxide producing capacity and the peroxidase activity of a sensitive and resistant strain was made, and some properties of the active peroxidase studied.

Methods. A trained resistant strain of *S. mitis* was grown aerobically and anaerobically to determine whether the peroxidase, if found, would be present only in the aerobic cells as reported by Seeley and VanDemark(3) for a strain of *S. faecalis*. Aerobic cultures were grown in liter flasks containing 100 ml of tryptose phosphate broth (TPB) through which air was bubbled. Anaerobic cultures were grown in 100 ml TPB in 125 ml flasks under a layer of mineral oil. After incubation at 37°C for 15-18 hours, cells were harvested, washed once and the crop from each flask suspended in 5 ml of water. One ml of each suspension was incubated at room temperature for one-half hour with 1 ml M/10 glucose, 1 ml H_2O_2 (75 μg), and 1 ml of water. After centrifugation, the supernatant was examined for residual H_2O_2 by the method of Main and Shinn(5).

Results. Aerobically grown cells destroyed all the H_2O_2, while 10% or less was destroyed

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† A portion of this work submitted in partial fulfillment of requirements for Ph.D. degree.

by those grown anaerobically. Repeated determinations gave the same results.

Boiled cells showed no destruction of H_2O_2. Living cells were inactive in the absence of glucose. An extract made by boiling the aerated cells in water was inactive but when added to living unaerated cells enabled them to destroy H_2O_2.

These results suggest that resistant *S. mitis* cells grown aerobically produce an enzyme which catalyzes the destruction of H_2O_2 in the presence of glucose. Part of this enzyme is heat stable and produced only by these cells, while another part is heat labile and is produced in both aerobic and anaerobic cultures. These reactions are similar to a peroxidase mechanism described by others(3,6) for a strain of *S. faecalis*.

Activity of peroxidase in sensitive and resistant cells. The foregoing experiments seemed to explain why H_2O_2 was not found in cultures of the resistant strain. However, it was of further interest to determine whether the resistant strain produced detectable amounts of H_2O_2 at any time during its growth, and whether the parent strain might produce a peroxidase but in a less active form. Before making these comparisons an attempt was made to determine optimum conditions for the activity of the peroxidase of the resistant strain. Variations of pH between 6 and 8, and growing the cultures and incubating the H_2O_2 with the cells at room temperature and 37°C, had little influence on the activity. Therefore, convenient and reproducible conditions were established by shaking the parent and resistant strains in a shallow layer of TPB for 15 to 18 hours at room temperature, and incubating the washed cells at 37°C in a buffered solution of pH 7. The activities of different cell concentrations of the two strains are shown in Table I. Both resistant and parent cells in very high concentrations were able to destroy all the added H_2O_2. However, at any lower concentration, the resistant cells only were active.

TABLE I. Disappearance of Added H_2O_2 Incubated with Equal Concentrations of Sensitive and Resistant *S. mitis* Cells.

Conc. of cells, Klett reading	% of H_2O_2 destroyed Resistant	Sensitive
>900*	100†	100
900	100	0
800	100	0
700	100	0
500	100	0

Quantities used: 1 ml each of cell suspension, M/10 glucose, H_2O_2 75 μg/ml, and pH 7 buffer. Incubation: 1 hr at 37°C.

* 1 to 10 dilution read 500.

† Values given for representative experiment. 3 to 5 determinations were made of each with no significant change.

Peroxide production of sensitive and resistant cells during growth. Peroxide production was measured at hourly intervals during growth of the two strains in TPB. Both strains produced peroxide during the first 12 hours of growth, but the amount produced by the resistant was much less than by the sensitive strain, and after 12 hours was destroyed. Repeated determinations gave essentially the same results.

Other properties of peroxidase. Resistant cells grown aerobically and anaerobically, inoculated into TPB and shaken continuously in both cases produced H_2O_2 in the early hours of growth. At 7 to 8 hours, both cultures began to show less H_2O_2, and none was found in either at 24 hours. With our resistant strain apparently a period of adaptation in the new aerated culture medium is necessary for both aerobically and anaerobically grown cells before the peroxidase is produced, or a period of growth is necessary to obtain enough cells for a constitutive peroxidase to show activity.

Sulfathiazole (2.5 mg/ml), penicillin (2,500 u/ml), and dihydrostreptomycin (250 μg/ml), when added to washed cells in the presence of H_2O_2, glucose, and buffer, did not interfere with the destruction of the H_2O_2. Dihydrostreptomycin, but not penicillin or sulfathiazole, did interfere with the peroxide destroying ability of a strain of *S. faecalis* tested at the same time.

Discussion. Non-proliferating cells of the trained resistant strain when harvested from an aerated culture showed peroxidase-like activity. No activity was found in cells obtained from anaerobic cultures, but they became active when an extract of the aerated cells was added. Similar activity of aerobically and anaerobically grown cells of *S. faecalis* was described by Seeley *et al.*(6). However, a difference in the time of the production of the peroxidase in growing cultures was observed by these investigators. They found no H_2O_2 in aerated cultures inoculated with previously aerated cells, but H_2O_2 was found early and then disappeared when the inoculation was made with previously unaerated cells. This was taken as evidence of an adaptive peroxidase produced only in the presence of oxygen. This difference was not observed in growing cultures of our resistant *S. mitis*.

The production of the more active peroxidase in the resistant cells apparently explains the lack of H_2O_2 in 24-hour and later cultures, since measurable amounts of H_2O_2 were produced in the early hours of growth by both sensitive and resistant strains. Whether this activity of the resistant cells contributes to their resistance, offering an alternate metabolic mechanism not developed in the sensitive strain is not evident from these experiments. The peroxidase was not inhibited by rather high concentrations of sulfathiazole, however. It was interesting that the activity was not inhibited by a concentration of dihydrostreptomycin, which did inhibit the peroxidase of a strain of *S. faecalis,* the growth of which was sensitive to less dihydrostreptomycin than the resistant *S. mitis*.

Summary. Aerobically grown sulfathiazole resistant cells of *S. mitis* produced a much more active peroxidase than the parent sensitive cells. H_2O_2 was produced by both the resistant and sensitive cells but was destroyed in the resistant cell culture after the first few hours of growth. Suspensions of anaerobically grown resistant cells had no peroxidase activity until a heat stable extract of the aerobically grown cells was added. The peroxidase activity was not appreciably changed by variation of pH between 6 and 8, change in temperature for growth or for incubation with H_2O_2, nor by the presence of sulfathiazole, penicillin or dihydrostreptomycin.

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Antagonism Studies on Reserpine and Certain CNS Depressants. (21458)

GRAHAM CHEN AND CHARLES R. ENSOR.
From Parke, Davis & Co., Detroit, Mich.

Reserpine has been shown in mice to possess a facilitation action on the central nervous system (a) by shortening the interval between the first clonic and the final tonic extensor response in Metrazol-induced convulsions, (b) by lowering the seizure threshold to electrical stimuli, and (c) by antagonism with Dilantin (1). Based on this pharmacological property of reserpine, the present study was designed to investigate possible mechanisms of action of various anticonvulsant agents. The results on antagonism between reserpine and 9 central nervous system depressants, as determined by electrically-induced hind leg extensor response in mice, are presented and elucidated in this report.

Methods. Male albino mice weighing 18-22 g were fasted 16 hours before oral administration; otherwise they were allowed free access to food and water. The tonic hind leg extensor seizure was induced by a 60 ∼ alternating current for 0.2 second(2). Thirty or forty mice, 10 per group, were used to determine the amperages of current which would be required to give a positive response in from 10 to 90% of the animals. The amperage of current prompting a response in 50% of the animals (CS_{50}) was estimated graphically by

TABLE I. Influence of Reserpine on Antielectroshock Effects of Various CNS Depressants in Mice. Reserpine = 5 mg/kg I.P. administered 5 hr prior to electroshock; 9 experiments.

Treatment	Dose effect							
D*	mg/kg OR 3 hr†	0	10	15	20	25	30	
Alone	CS$_{50}$ ± S.E. m.a.	4.7±.05	9.9± .5	123 ±68	>400			
+ R	” ” ”	3.7±.11	4.8± .1	4.8± .2	7.6± .7	48 ±19	250±92	
PM 396	mg/kg OR 45′		80	90	100	140	160	180
Alone	CS$_{50}$ ± S.E. m.a.		18 ± .3	93 ±15	>400			
+ R	” ” ”		4.7± .4	5.1± .3	6.4± .4	17 ± 2	65±17	183±19
Ph	mg/kg IP 3 hr		16	24	32	40	50	60
Alone	CS$_{50}$ ± S.E. m.a.		7.1± .2	14 ± 1.6	87 ± 3.4	—	—	—
+ R	” ” ”		4.7± .1	5.9± .3	6.8± .3	8.8± .3	57±11	200±37
M	mg/kg OR 45′		120	150	180	210	240	270
Alone	CS$_{50}$ ± S.E. m.a.		6.6± .3	20 ± 4.8	87 ±33	—	—	—
+ R	” ” ”		—	5.9± .3	10 ± .7	21 ± 5	116±35	172±41
Phe	mg/kg OR 1 hr		90	110	130	160	190	220
Alone	CS$_{50}$ ± S.E. m.a.		8.6± .2	33 ±10	222 ±63	—	—	—
+ R	” ” ”		—	5.7± .2	13 ± 2	32 ± 7	35± 4	86±24
Car	mg/kg OR 1 hr		140	160	180	200	220	240
Alone	CS$_{50}$ ± S.E. m.a.		7.2± .3	20 ± 2	>400			
+ R	” ” ”		6.2± .3	7 ± .3	14 ± 1	16 ± 1	120±32	164±16
Bar	mg/kg IP 1 hr		220	240	260	280	310	340
Alone	CS$_{50}$ ± S.E. m.a.		12.4±1.4	54 ± 7.6	119 ±35	—	—	—
+ R	” ” ”		7.0± .3	9.0± .2	14 ± .4	19 ± .3	42± 8	89± 6
Meph	mg/kg IP 10′		70	100	130	140	150	
Alone	CS$_{50}$ ± S.E. m.a.		5.2± .1	33 ±10	>400	—	—	
+ R	” ” ”		4.8± .1	11.6± 1.0	96 ±27	156 ±39	>400	
Pent	mg/kg IP 15′		28	32	36	40	45	
Alone	CS$_{50}$ ± S.E. m.a.		8.1± .2	28 ± 1.4	245 ±57	—	—	
+ R	” ” ”		5.2± .1	6.7± .3	20 ± 5	49 ± 8.3	245±45	

* D = Dilantin; R = Reserpine; Ph = Phenobarbital; M = Milontin; Phe = Phenacemide; Car = Carbromal; Bar = Barbital; Meph = Mephenesin; Pent = Pentobarbital.

† Time after administration.

the probit and log-amperage relationship(3). *Reserpine* was finely suspended in gum acacia solution for intraperitoneal administration. In one series of experiments, a fixed dose of reserpine (5 mg/kg) and different doses of an anticonvulsant were used. Five hours were allowed for the onset of the facilitating effect of reserpine prior to application of electroshock (1). In another series of experiments, when different doses of reserpine were titrated against different doses of an anticonvulsant, the animals were shocked 20-24 hours after reserpine administration. The anticonvulsant effect of a compound was examined at the time of its peak effect, the latter having been previously determined. The central *nervous system depressants* included in our study were: sodium 5, 5-diphenyl hydantoin (Dilantin sodium); sodium 5-ethyl-5-phenyl barbiturate (phenobarbital sodium); sodium 5, 5-diethyl barbiturate (sodium barbital); sodium 5-ethyl-5(1-methylbutyl) barbiturate (pentobarbital sodium); N-methyl-α-phenylsuccinimide (Milontin); N-methyl-α-methyl-α-phenylsuccinimide (PM 396); phenylacetylurea (phenacemide); bromo-diethylacetylurea (carbromal); and 3-(o-toloxy)-1,2-propanediol (mephenesin). They were chosen as representative therapeutic agents for the management of grand mal and petit mal epilepsy, psychomotor seizures, for sedation and hypnosis, and for general anesthesia.

Results. In Table I are recorded the results obtained in experiments with 5 mg/kg of reserpine and different doses of depressants. The current strength (CS$_{50}$) listed first was determined for the animals receiving only the CNS depressant; the second value in each group was determined for animals receiving both CNS depressant and reserpine. The influence

TABLE II. Comparison of Antielectroshock Effects of Various CNS Depressants under Influence of Reserpine (5 mg/kg IP) in Mice with a Current of 20 m.a. (from Data in Table I).

Treatment	Symptoms	Antielectroshock dosage mg/kg	Diff.	% diff.	Order of antagonism by reserpine
Dilantin	0	11	11	100	1‡
+ Reserpine	0	22			
PM 396	0	80	62	77.4	2
+ Reserpine	L.*	142			
Phenobarbital	0	25	17	68.0	3
+ Reserpine	L.	42			
Milontin	0	150	55	36.7	4
+ Reserpine	Loss of G.R.†	205			
Phenacemide	0	110	38	34.6	5
+ Reserpine	L.	148			
Carbromal	L.	160	45	28.1	6
+ Reserpine	Loss of G.R.	205			
Barbital	L.	222	58	26.1	7
+ Reserpine	Loss of R.R.†	280			
Mephenesin	Weak	88	16	18.2	8
+ Reserpine	"	104			
Pentobarbital	Ataxic	31	5	16.1	9
+ Reserpine	Loss of G.R.	36			

* L. = Lethargic. † R.R. = Righting reflex; G.R. = Grasping reflex. ‡ Most effective (greatest % increment of antielectroshock dosage).

of reserpine on the anticonvulsant effect of each agent is best shown from the data by examining the increment in anticonvulsant dosage for reserpine-treated animals as compared to untreated controls. For instance, the anticonvulsant dosages of Dilantin for a stimulus of 20 m. a. (by interpolation graphically) were 11 and 22 mg/kg without and under the influence of reserpine respectively, an increment of 100%. In the case of barbital, on the other hand, the dosages under the same stimulus were 222 and 280 mg/kg without and with reserpine respectively, an increment of 26.1%. In other words, reserpine is more effective in antagonizing the anti-electroshock effect of Dilantin than it is that of barbital. The increments so calculated at a stimulus of 20 m. a. for the nine depressants and their relative effectiveness in reserpine antagonism are given in Table II. In decreasing order they are: Dilantin, PM 396, phenobarbital, Milontin, phenacemide, carbromal, barbital, pentobarbital, and mephenesin. Another feature which may be pointed out from the data in Table II is that, whereas their anticonvulsant effects were greater when they were given without than with reserpine, the animals were more deeply depressed under the influence of reserpine. In other words, the results indicate a dual central action of reserpine: a depressant action symptomatically and a facilitary action by the hind leg extension response.

The quantitative differences in antagonism dosages between reserpine and the various depressants led us to carry out another series of experiments in which the doses of both reserpine and the depressant were varied and their effect on electroshock determined at a constant stimulus of 20 m. a. This current strength was chosen because it is at the part of the dose-response curve having a maximal rate of change in slope and thus offers a greater sensitivity for examining the effect of dosage on electroshock. In Table III are presented the results obtained with reserpine against Dilantin, phenobarbital, PM 396, barbital, Milontin, carbromal, mephenesin, and a combination of Dilantin and barbital. At the top of the table are listed the doses of reserpine in a geometrical series with a factor of 2. The figures below for each depressant are ED_{50} values or the doses required (together with reserpine) to suppress the tonic hind leg extensor response in 50% of the mice. There are included in the

TABLE III. Antagonism between Reserpine and Various CNS Depressants as Determined in Mice with a Convulsive Stimulus of 20 m.a.; 9 Experiments.

Depressant	Reserpine, mg/kg (IP, 24 hr)	0 (control)	.25	.50	1.0	2.0	4.0
Dilantin (IP 3 hr)	ED$_{50}$ ± S.E., mg/kg a	13.5 ± .25	15.1 ± .24	16.9 ± .32	20.1 ± .42	25.5 ± .49	31.0 ± .97
	Diff. from control b		1.6 ± .32	3.4 ± .42	6.6 ± .59	12.0 ± .61	17.5 ± 1.0
	Diff. between doses c			1.8 ± .5	3.2 ± .7	5.4 ± .7	5.5 ± 1.2
Phenobarbital (IP 3 hr)	a	31.5 ± 1.0	36.5 ± .5	40.0 ± 1.0	48 ± .7	58 ± .6	61 ± .9
	b		5.0 ± 1.1	8.5 ± 1.4	16.5 ± 1.2	26.5 ± 1.2	29.5 ± 1.3
	c			3.5 ± 1.7	8.0 ± 1.9	10.0 ± 1.7	3.0 ± 1.8
PM 396 (IP 1 hr)	a	76.0 ± 2.7	86.3 ± 2.4	102 ± 1.7	117 ± 25	132 ± 2.9	143 ± 2.2
	b		10.3 ± 3.6	26 ± 2.9	41 ± 3.0	56 ± 3.8	67 ± 3.6
	c			15.7 ± 5.1	15.0 ± 4.2	15.0 ± 4.7	11.0 ± 4.8
Barbital (IP 1 hr)	a	200 ± 4.3	232 ± 3.8	237 ± 3.7	264 ± 2.8	280 ± 2.7	282 ± 3.9
	b		12 ± 5.7	17 ± 5.3	64 ± 4.6	80 ± 3.9	82 ± 4.7
	c			5 ± 7.8	47 ± 7.1	16 ± 6.1	2 ± 6.1
Milontin (IP 45′)	a	142 ± 2.9	167 ± 3.2	165 ± 2.0	190 ± 3.6	222 ± 3.5	235 ± 5.3
	b		25 ± 4.3	23 ± 3.8	48 ± 4.1	80 ± 5.0	93 ± 6.4
	c			−2 ± 5.7	25 ± 5.6	32 ± 6.5	13 ± 8.1
Carbromal (IP 45′)	a	164 ± 2.4	168 ± 2.4	175 ± 2.0	192 ± 3.4	212 ± 2.5	185 ± 2.0
	b		4 ± 3.4	11 ± 3.1	28 ± 4.2	48 ± 3.5	21 ± 3.1
	c			7 ± 4.6	17 ± 5.2	20 ± 5.5	-21 ± 4.7
Mephenesin (IP 10′)	a	108 ± 1.8	105 ± 1.7	108 ± 1.8	113 ± 3.1	121 ± 4.3	123 ± 2.7
	b		−3 ± 2.5	0 ± 2.5	5 ± 3.6	13 ± 5.3	15 ± 5.1
	c				5 ± 4.4	8 ± 6.4	2 ± 7.4
Dilantin	a	9.8 ± 0.23	11.3 ± .24	12.0 ± .24	12.9 ± .15	13.5 ± .15	12.5 ± .19
	b		1.5 ± .3	2.2 ± .3	3.1 ± .3	3.7 ± .3	2.7 ± .3
+	c			.7 ± .4	.9 ± .4	.6 ± .4	−1.0 ± .4
Barbital (IP 3 hr)	a	130 ± 3.3	151 ± 3.2	160 ± 3.2	172 ± 2.1	180 ± 2.0	166 ± 2.6
	b		21 ± 4.5	30 ± 4.5	42 ± 3.9	50 ± 3.9	36 ± 4.2
	c			9 ± 6.4	12 ± 5.9	8 ± 5.5	−14 ± 5.7

TABLE IV. Empirical Formulae and Values of Constants Estimated Graphically from Data in Table III.

		Value of constant in micromols		
Antagonism*	Formula used	a	b	c
R + D	$y = a + bx^c$	.10	.0686	1.02
"	"	.22	.0426	1.04
R + Ph	$y = a + bc^x$	0	.240	1.026
"	"	.10	.225	1.029
R + PM 396	"	−.10	.218	1.007
"	"	0	.255	1.009
R + D + B	"	.12	.128	1.247D
		"	"	1.013B
"	"	−.10	.100	1.291D
		"	"	1.014B

* R = Reserpine; D = Dilantin; Ph = Phenobarbital; B = Barbital.

table two sets of values calculated from the ED_{50}'s for each compound: (a) ED_{50} differences between the control result (without reserpine) and the result recorded with reserpine, and (b) ED_{50} differences at successive doses of reserpine. The former figures represent quantities of the depressant, the anti-electroshock effect of which was abolished by corresponding quantities of reserpine. The latter are given to show the dose relationship between the two antagonists.

It may be seen that the antagonistic doses of Dilantin, like the reserpine dosages, form a geometrical series (an average factor of 1.95). Empirically, when 2 variables, x and y, are so related in a geometrical series, the relation between the 2 variables may be expressed by the equation, $y = a + bx^c$. Table IV gives the values of constants a, b, c in micromols for such a relation when applied to reserpine (y) and Dilantin dosages (x). The values were estimated graphically from the data in Table III and from the results of another experiment(4). When $x = o$, $y = a$; the constant "a" may be considered a threshold dose of reserpine. It amounts to 0.10 to 0.22 micromols or 0.06 to 0.12 mg/kg. Since this value is small in comparison with the reserpine doses employed in the experiment, an approximately linear relationship is shown by plotting the antagonistic doses of reserpine and Dilantin in logarithms ($y = bx^c$, or $\log y = \log b + c \log x$). The equation, $y = a + bx^c$, is similar to the formula, $C_1 = \frac{1}{K}\,[1 + (K_2C_2)^n]\,\frac{a}{1-a}$ proposed by Gaddum for competitive inhibition(5). Since in our experiment the different antagonistic doses of reserpine and Dilantin were determined at a response for 50% of the mice to a constant stimulus of 20 m. a., the term "*a*" representing the proportion of the receptors engaged in the reaction is the same for all determinations. Gaddum's formula thus becomes $C_1 = K_3 + K_4C_2^n$, where C_1 and C_2 are concentrations of the antagonists, and K_3, K_4, and n are constants. The equation has been shown to fit the data on antagonism between sulfonamide and para-aminobenzoic acid(6,7). The antagonism between reserpine and Dilantin appears therefore to be competitive in nature.

The above formula was not applicable to the data obtained with reserpine and other CNS depressants in Table III. In the case of phenobarbital or PM 396, antagonistic doses fall approximately into an arithmetical series, which is clearly indicated by the differences in ED_{50} for PM 396 (15 mg/kg) between successive doses of reserpine. Empirically, when x is in an arithmetical series while y is in a geometrical series, the relation between the two variables is expressed by the equation, $y = a + bc^x$. From the data in Table III and the results of 2 additional experiments, the values of the constants a, b, and c were estimated graphically for the relationship between reserpine (y) and phenobarbital (x), and between reserpine and PM 396. Since $a = o$ for the 2 experiments, the data are fitted as well by the simpler formula: $y = bc^x$, or $\log y = \log b + x \log c$. This gives a straight line relating the logarithmic doses of reserpine and the corresponding doses of phenobarbital (or PM 396). Some of the features which are suggested by the equation, $y = a + bc^x$, are: (a) when $x = o$, $y = a + b$ which may be looked upon as a threshold dose of reserpine. (b) The value "b" is approximately the same for phenobarbital and PM 396 (0.218 — 0.255), implying that the 2 agents produce an anticonvulsant effect apparently by the same mechanisms. (c) The magnitude of the constant "c" is in direct proportion to the activity of an anticonvulsant. The above propositions, as derived from the empirical formulae for reserpine, Dilantin, and phenobarbital are aimed primarily at uncovering certain possible mechanisms by which these compounds produce their anticonvulsant effects. They also show how anticonvulsant effects may be quantitatively evaluated. Other investigators may find that the data are fitted better to a different formula and may offer a different interpretation of reserpine antagonism toward Dilantin or phenobarbital. The reader should, therefore, look upon our empirical approach merely as a tool for studying the anticonvulsant action of a compound, and should realize the limitations of applying mathematics to biological data(8).

A simple solution has not been found to relate the antagonistic dose of reserpine with that of Milontin, barbital, or carbromal. The data indicate, however, that the antagonism between reserpine and these compounds is much less pronounced than that between reserpine and phenobarbital or PM 396. For 0.25 to 0.5 mg/kg of reserpine, the difference between antagonistic doses of Milontin, barbital, or carbromal is not statistically significant. The antagonistic dose of carbromal for 4 mg/kg of reserpine is less than that for 2 mg/kg of reserpine. The apparent paradox may be due to an antielectroshock effect of reserpine itself at 4 mg/kg. The central depressive effect of reserpine appears to be more marked when examined in conjunction with the anticonvulsant effect of another agent. It was also obtained when the drug was investigated jointly with carbromal and other CNS depressants in strychnine-induced convulsions(9).

Reserpine is least effective in antagonizing the antielectroshock effect of Mephenesin, antagonism being noticeable only at high reserpine dosages (2 and 4 mg/kg). Since the action of reserpine in these experiments is to facilitate the discharge of convulsive seizures and since reserpine and Dilantin appear to antagonize competitively, the mode of the anticonvulsant action of Dilantin is apparently to impede the spread of seizure discharge. This supports the suggestion of Toman and Goodman that the anticonvulsant effect of Dilantin is primarily due to an inhibition of seizure spread(10).

In order to find an explanation for the anticonvulsant effect of other CNS depressants, as shown by the data in Tables II and III, the antagonism between reserpine and a combination of Dilantin and barbital was also investigated. The assumption was made that the anticonvulsant action of barbital is different from that of Dilantin. Barbital probably raises principally the threshold for convulsive discharge. The combination of Dilantin and barbital would then produce an anticonvulsant effect both by inhibiting seizure spread and by raising seizure threshold. For such a study, the doses of the 2 compounds were made up proportional to their individual control ED_{50}'s (15 mg/kg of Dilantin and 200 mg/kg of barbital). The combination was administered intraperitoneally in one solution 3 hours prior to ED_{50} determinations. As the data in Table III reveal, the ED_{50} values either in terms of Dilantin or barbital in the mixture form approximately an arithmetical series from 0.25 to 2.0 mg/kg of reserpine. The antagonism between reserpine and the combination (*i.e.* in terms of Dilantin or barbital dosage) is thus related by the same equation, $y = a + bc^x$, for antagonism between reserpine and phenobarbital or PM 396. The values of the constants a, b, c calculated on the basis of the relationship between reserpine dosage and Dilantin or barbital dosage in the mixture, are given in Table IV from the results of 2 experiments.

The quantitative relationship in antagonism between reserpine and the combination of Dilantin and barbital suggests that the antielectroshock effect of phenobarbital or PM 396 is due to 2 anticonvulsant mechanisms, inhibition of seizure spread and elevation of seizure threshold. The difference in the degree of antagonism between reserpine and the various CNS depressants, as indicated by the data in Tables II and III, signifies then a difference in the extent to which the two mechanisms are involved in their anticonvulsant effects. Evidently, in addition to anticonvulsant mechanisms, the site of action should also be considered in interpreting the antagonism data. The insignificant antagonism between reserpine and mephenesin is probably due to the different sites on which the 2 act in the central nervous system.

Summary. 1. Quantitative studies with electrically induced convulsions have been carried out in mice on antagonism between reserpine and the following CNS depressants—Dilantin, N-methyl-α-methyl-α-phenylsuccinimide (PM 396), phenobarbital, Milontin, phenacemide, carbromal, barbital, pentobarbital, and mephenesin. 2. Antagonism between reserpine and Dilantin appears to be competitive in nature, a facilitatory and an inhibitory effect respectively, acting on the spread of seizure discharge. 3. Antagonism data for reserpine and phenobarbital as well as those for reserpine and PM 396 were found to bear a relationship similar to that between reserpine and a combination of Dilantin and barbital, indicating a dual mechanism in their anticonvulsant effect, inhibition of seizure spread and elevation of the threshold for seizure discharge. 4. Quantitative differences in antagonism between reserpine and the various CNS depressants are apparently due to differences in the extent to which the two mechanisms are involved in their anticonvulsant effects.

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Effect of 2-methyl, 2-*n*-propyl-1,3-propanediol dicarbamate (Miltown) on Central Nervous System. (21459)

C. D. HENDLEY, T. E. LYNES, AND F. M. BERGER.

From Wallace Laboratories, Division of Carter Products, Inc., New Brunswick, N. J.

The 2,2-substituted 1,3 propanediols investigated during a survey of compounds related to mephenesin proved of particular interest because of their influence on higher levels of the central nervous system (CNS) than mephenesin(1). The dicarbamate of 2-methyl-2-*n*-propyl-1,3-propanediol (Miltown) was the most potent anticonvulsant in this series, and also possessed a muscle relaxant action of considerable intensity and long duration(2,3). The present report is concerned with the effect of this compound on spinal reflexes and on the electrical activity of higher levels of the CNS.

Methods. Spinal and bulbar reflexes were elicited in 13 cats lightly anesthetized with chloralose-urethane or Dial, using methods previously described(3,4). The electrical activity of the brain was studied in 6 male cats. The animals were placed in a stereotaxic apparatus and prepared for recording under ether and local anesthesia. Solder electrodes were placed epidurally over cortical areas and a bipolar nichrome electrode, with 1 mm. separation, was located in the thalamus at Horsley-Clarke coordinates A12, R4, HO. Electrical potentials were recorded with a Grass Model 3D encephalograph. The animals were immobilized with d-tubocurarine chloride, and 2 hours allowed for the effects of ether to wear off, before starting the experiment. Miltown was given slowly by the intravenous route in 1% supersaturated solution in saline.

Results. As shown in Table I, Miltown profoundly reduced amplitude of the flexor reflex

TABLE I. Effect of Miltown on Spinal and Bulbar Reflexes of 16 Cats.

Dose, mg/kg	Amplitude of reflex response as percent of control		
	Knee-jerk	Flexor reflex	Linguo-mandibular reflex
20	100	100	
30	”	70	
”	”	0	
”	”	40	
”	”	0	
”	”	60	
40	”	0	
”	80	0	
”	”		0
”	110		100
”	125		100
50	100		
”	90		25
”	100		0
80	”		0
”	”		0

at a dose of 30 mg/kg, and similarly affected the linguo-mandibular reflex at somewhat higher doses. During blockade, reflexes could be re-elicited by increasing the intensity of stimulus. Amplitude and briskness of the knee jerk were not usually affected, even by large doses of the compound (50-80 mg/kg). In certain animals the administration of Miltown resulted in a slight increase in amplitude of the knee jerk, which may have been due to a relaxation of hypertonic skeletal muscle. Occasionally a slight decrease was noted, possibly resulting from a marked loss in muscle tone brought about by injection of Miltown in more deeply anesthetized animals.

Miltown caused definite and reproducible changes in electrical activity of the brain, as illustrated in Fig. 1. Before the drug, a typical waking record was obtained, with comparatively low voltage fast activity. The dominant frequency in both cortical and thalamic leads was 25-30/sec. The maximum voltage in the cortex was about 100 μV, and in the thalamus, 15 μV. Following the intravenous injection of 40 mg/kg Miltown in divided doses, there was a marked synchronization in the record. The cortical voltage was more than doubled, while in the thalamic leads it was quadrupled. This effect was seen to a lesser degree in all the cats after a single dose of 20 mg/kg. Changes in the record usually appeared immediately after injection of Miltown (injection time was 0.5 to 2 minutes), but there was a delay of 5 to 30 minutes before the effects were fully developed, and they persisted for over an hour thereafter.

The effects of Miltown were most pronounced in the thalamic leads, and usually appeared there before cortical changes occurred. Occasionally, 8-10/sec. spindling was seen in all leads, and here again the thalamus seemed to be affected first.

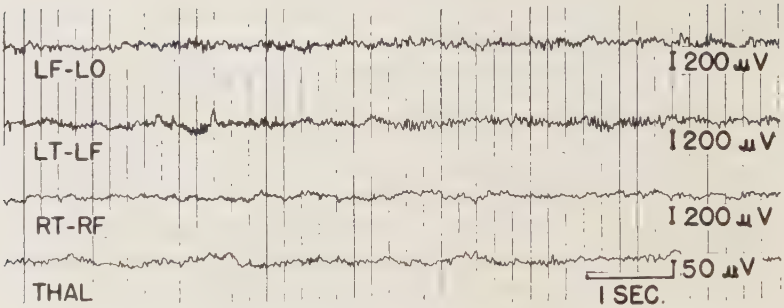
In animals with dominant cortical frequencies of 30-40/sec., Miltown at a dose of 20 mg/kg caused a slowing to about 20-22/sec. With very large doses (80-120 mg/kg) high voltage slow activity (from 8 down to 1/sec.) appeared, and a record characteristic of sleep was obtained.

It was interesting to note that at the time Miltown was first given the cats were usually recovering from the effects of d-tubocurarine and were beginning to show voluntary activity. A single dose of 20 mg/kg Miltown quieted and relaxed the animals. Later, as further doses of Miltown were given, no additional curarization was ever necessary.

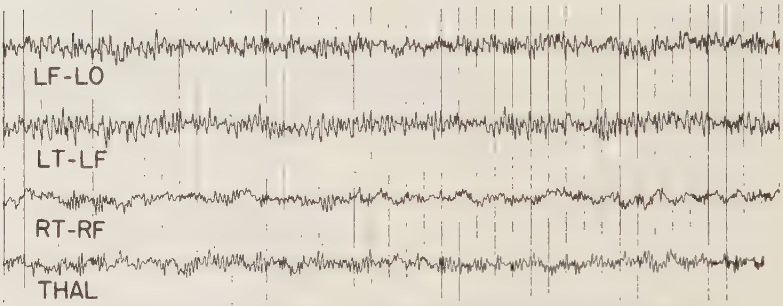
Discussion. In the spinal cord Miltown appears to have an effect similar to that of mephenesin. This can be explained by a blocking action of the drug on the interneurons interposed between the afferent and efferent arc of multineuronal reflexes. Mephenesin and Miltown differ strikingly from each other in their effect on brain waves. Mephenesin is known to have no effect on normal EEG patterns in doses that abolish involuntary movements or tremors(5). Miltown, on the other hand, has a marked effect on the EEG, causing a marked synchronization, with a moderate slowing of frequency. This action of the drug is most apparent in the diencephalon.

The asynchronous activity responsible for the normal waking record may be dependent on the integrity of long interneuronal circuits. The blockade of the more complex of these by Miltown would permit the emergence of a more synchronous activity.

The greater influence of Miltown on the thalamic record may be due to a preferential



3:29 P.M. BEFORE MILTOWN



3:52 P.M. MILTOWN 20 MGM/KGM I.V. AT 3:30 P.M. AND AT 3:47 P.M.

FIG. 1. Effect of Miltown on cortical and thalamic electrical activity. Bipolar recordings from left frontal (LF), left temporal (LT), left occipital (LO), right frontal (RF), right temporal (RT), and right ventral thalamic (THAL) regions.

effect on cells of the thalamus. Alternatively the removal of cortical influences may permit the emergence of an intrinsic thalamic rhythm.

Summary. 2-methyl, 2-*n*-propyl, 1,3 propanediol dicarbamate has been shown to have a potent and persistent mephenesin-like action on multineuronal spinal and bulbar reflexes in the cat. Recordings of electrical activity of the brain show that this compound in low doses will cause increase in voltage and decrease in frequency. This effect is more apparent in recordings from the diencephalon than in those from the cerebral cortex.

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Reactivation of Serum Neutralized Influenza Virus and Failure of its Use in Primary Isolation. (21460)

Kenneth K. Takemoto,[†] Paul Beigelman,[‡] and Dorland J. Davis.

From the National Institutes of Health, National Microbiological Institute, Bethesda, Maryland.*

In connection with epidemiological studies of influenza based upon specific diagnosis by virus isolation or serological tests, we have explored methods for more efficient isolation of virus from throat swabs and washings. One of these was a result of the suggestion by McKee and Hale(1) that influenza virus may not be recovered from human nose and throat washings due to the presence of antibody, and that the addition of inactive virus may serve as a means for releasing antibody-bound virus. In their studies(1-3), McKee and associates have shown that neutralized influenza virus can be reactivated by the addition of concentrated, heat-inactivated virus. In a later study(4) they reported the failure to recover influenza virus from nose and throat washings after adding concentrated inactive virus to the washings.

Our studies include attempts at virus isolation through the use of inactive virus on 106 samples from patients having influenza-like illness. We have also confirmed and extended the studies of McKee and associates on reactivation of neutralized mixtures of influenza, using 4 type A and 2 type B strains of influenza.

Materials and methods. Preparation of active and inactive virus. Four type A influenza strains, A/PR8, A/FW/1/50 (Cuppett), A/FLW/1/52 (Conley), and A/Va/1/53, and 2 type B strains, B/Lee and B/Va/1/50, were used. All strains were egg-adapted and had been passed many times in chick embryos. Fresh suspensions of each strain were prepared by intraallantoic inoculation of 0.2 ml of a 10^{-3} dilution of seed virus. After 48 hours incubation, allantoic fluid from living eggs was harvested, pooled and titrated for infectivity. A part of each pool was stored as active virus in 0.5 ml amounts in a dry ice box and the remainder inactivated at 56°C for 80 minutes. Preliminary experiments confirmed the work by McKee and Hale(2) that PR8 virus loses both its hemagglutinating and antibody combining capacities when heated at 56° C for 80 minutes, and inactive PR8 prepared in this manner was ineffective as a reactivating agent. Inactive PR8 virus was, therefore, obtained by exposure of infected allantoic fluid to a 25 watt General Electric germicidal lamp by continuous flow in an apparatus similar to that described by Habel and Sockrider(5). The infectious allantoic fluid was allowed to flow through the apparatus in a thin film at the rate of 5 ml/min. The fluid was then heated for 15 minutes at 56°C to inactivate residual active virus. All inactive virus preparations were tested for presence of active virus by three serial passages in 11-day-old chick embryos and hemagglutination tests were employed to detect virus in allantoic fluids. *Immune sera* were obtained from roosters after one 5 ml intravenous and one 10 ml intraperitoneal inoculation of virus infected allantoic fluid. Prior to use, sera were inactivated by heating at 56° C for 30 minutes. *Reactivation experiments.* To test for reactivation, serial 10-fold dilutions of active virus were made in bacteriological broth. An equal volume of diluted immune serum was added to each tube and the mixtures kept at room temperature for 15 minutes. Inactive homologous or heterologous virus equal in volume to the active virus was then added to the tubes, the mixtures shaken, kept at room temperature for 15 minutes, then at 4°C until inoculated. For controls, one set of tubes containing serially diluted active virus and immune serum were mixed with normal allantoic fluid from 13-day embryos heated at 56°C for 80 minutes. Three-tenths ml of the mixtures were inoculated intra-allantoically

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* Laboratory of Infectious Diseases.

[†] Post-doctoral Fellow of the National Microbiological Institute, National Institutes of Health.

[‡] Present address, Peter Bent Brigham Hospital, Boston, Mass.

TABLE I. Titer and Difference in Logs of 6 Strains of Influenza Virus Neutralized by Homologous Antiserum and Reactivated by Inactive Homologous and Heterologous Virus.

	Active virus					
	A/PR8	A/FW/1/50	A/FLW/1/52	A/Va/1/53	B/Lee	B/Va/1/50
H.A.* + normal All. Fl.	2.49	1.16	4.16	5.12	1.50	1.16
H.A. + inact. A/PR8	6.16† +3.67	1.61 + .45	4.85 + .69	5.47 + .35	1.83 + .33	1.51 + .35
H.A. + inact. A/FW/1/50	3.14 + .65	3.64 +2.44	4.66 + .50	5.53 + .41	1.50 0	1.16 0
H.A. + inact. A/FLW/1/52	2.75 + .26	1.63 + .47	7.37 +3.21	7.25 +2.13	1.75 + .25	1.28 + .12
H.A. + inact. A/Va/1/53	2.94 + .45	1.50 + .34	7.16 +3.00	7.16 +2.04	1.52 + .02	1.16 0
H.A. + inact. B/Lee	2.75 + .26	1.33 + .17	4.75 + .59	5.25 + .13	4.25 +2.75	1.61 + .45
H.A. + inact. B/Va/1/50	2.84 + .35	1.50 + .34	4.60 + .44	5.50 + .38	1.57 + .07	3.30 +2.14

* H.A. = Homologous antiserum.

† Upper figures are the 50% end-points. Lower figures show log increase due to addition of inactive virus.

into 11-day-old chick embryos, 5 eggs per dilution. After 48 hour incubation, eggs were chilled and allantoic fluids tested for hemagglutination. Fifty percent end-points were calculated by the Reed and Muench method (6). Preliminary experiments were done with each immune serum to determine the serum dilution which would give the greatest degree of reactivation in the presence of homologous active and inactive virus, and this optimum dilution was used in all reactivation experiments. The experiments on influence of time of addition of inactive virus to neutralized mixtures were conducted under similar conditions. The active virus dilutions and antiserum were mixed and allowed to remain at room temperatures for 15 minutes, then kept at 4°C. At various time intervals, inactive virus was added and the tubes kept at room temperature for 30 minutes before inoculation. *Virus isolation with inactive virus.* Throat swabs and washings in skim milk were obtained during the winter epidemic of 1953 and stored in a dry ice box until used. Strain A/Va/1/53, isolated at beginning of the epidemic from which samples were collected, was concentrated by centrifugation of infected allantoic fluid at 13,500 rpm for 30 minutes, after which the supernate was decanted and sediment reconstituted with sterile saline in one tenth original volume. Infectivity titer was increased 10 to 100 fold by this method. Virus was inactivated by heating in a 56°C water bath for 80 minutes and its noninfectiousness confirmed by 3 serial passages in 11-day-old chick embryos. Equal volumes of throat washings or swabs samples and concentrated, inactive virus were mixed and kept at room temperature for 2 hours after which 11-day-old chick embryos were inoculated intra-amniotically with 0.2 ml of the mixture. After 5 days incubation at 37°C, lungs and fluids were harvested, ground in alundum, and re-inoculated intra-amniotically into another group of 11-day-old embryos. After 48 hours incubation, amniotic and allantoic fluids were tested for hemagglutination with 0.4% chicken red blood cells.

Results. Table I shows the results of reactivation experiments with 6 strains of influenza virus. When homologous inactive virus was added to active virus-antibody mixtures, a 100 fold or greater increase in virus titers was demonstrated with all strains. There appears to be no difference between A/Va/1/53 and A/FLW/1/52 viruses, and each is capable of reactivating the other to the same degree. Further investigations of these 2 strains by cross hemagglutination-inhibition and cross-neutralization experiments showed no anti-

TABLE II. Influence of Time of Addition of Inactive Virus on Reactivation.

Active virus	Titer with antiserum	Titer after inactive homologous virus added at							
		15 min.	1 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr
B/Lee	2.5	5.33	3.52	3.37	3.16	2.76	—	—	—
A/Va/1/53	3.74	6.16	—	—	—	4.95	4.75	4.33	3.85

genic differences. The other type A strains, A/FW/1/50 and A/PR8, failed to cross-react with the other viruses. The 2 type B strains, B/Lee and B/Va/1/50, did not reactivate the A strains. Although the type B strains show some degree of antigenic similarity by hemagglutination-inhibition and neutralization tests, this similarity is apparently not great enough to cause one virus to reactivate the other. Reactivation of neutralized influenza virus appears to be serologically quite strain specific.

The influence of time of addition of inactive virus to homologous active virus-antiserum mixtures is shown in Table II. With Lee virus, a steady decline in the degree of reactivation is noted until union of active virus and antibody becomes irreversible at the end of 4 hours. With the A/Va/1/53 virus, a 10-fold increase can still be noted at the end of 6 hours, but no reactivation is observed at the end of 10 hours. Hultin and McKee(3) have reported that when inactive virus is injected into eggs previously inoculated with neutralized virus, reactivation occurred up to 24 hours.

Primary virus isolations with the use of concentrated, inactive virus were attempted on 106 throat washings and swabs. In the first group of 11 samples, virus had been previously isolated without the use of inactive virus. When tested with the addition of concentrated, heat-inactivated virus, influenza virus was recovered from all but 2 samples. When these 2 were retested a second time without inactive virus, they were found to be positive. Although no evidence of interference was noted in the reactivation studies with the 6 egg-adapted strains of influenza, it is possible that such a phenomenon may explain the failure to recover virus in these 2 samples.

Of the 95 samples in the second group which were negative when tested without inactive virus, only one new isolation was obtained. Virus was also isolated from this sample when tested again without inactive virus. The failure to recover influenza virus from these samples may be due to several possibilities. If antibodies were present with virus and were the interfering agents, an irreversible union may have taken place before the addition of inactive virus. It is also probable that influenza virus was not present in these samples.

Summary. 1. Inactivated viruses of 4 strains of influenza A and 2 strains of influenza B reactivated serum neutralized homologous virus with an increase in titer of 100 fold or more. The reaction is serologically strain specific. 2. The degree of reactivation is dependent on time of addition of inactivated virus to active virus-antiserum mixture. The threshold time after which reactivation could not be noted was about 8 hours for A/Va/1/53 and about 3 hours for B/Lee. 3. Addition of concentrated, heat-inactivated virus to samples from throats of persons ill with influenza-like illness during an influenza epidemic failed to increase the number of influenza virus isolations over the usual method.

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Reserpine Antagonism of Morphine Analgesia in Mice. (21461)

JURG A. SCHNEIDER. (Introduced by Robert Gaunt.)
(With the technical assistance of Jacqueline van de Kamp and Harold Coombs.)
From the Research Department, CIBA Pharmaceutical Products, Inc., Summit, N. J.

Reserpine (Serpasil), an effective alkaloid of *Rawolfia serpentina* Benth (Muller, Schlittler and Bein(1)) has been widely used as a drug having a calming effect in psychiatric disorders (*e.g.* Kline(2)). Its ability to exert a tranquilizing influence on patients without causing anesthesia (Noce(3)) proved to be one of its most interesting features. Although reserpine produces a condition characteristic of general parasympathetic predominance (Bein(4)), it has no analgetic effect (Bein, Gross, Tripod and Meier(5)). Because of its point of attack in the central nervous system (4,5) it was, however, of interest to check whether reserpine interfered in any way with the analgetic action of morphine. Chloropromazine was included in this study because it is used in similar psychiatric conditions as reserpine and a comparison of the two drugs seemed to be indicated. Results of such a study should be of importance both from a practical and theoretical point of view.

Material and methods. White mice (Webster) of both sexes weighing approximately 20 g were used in all experiments. In each experiment 10 mice were placed in small individual cylindrical cages with their tails extending from the ends of the cages. A beam of heat was focused on the tip of the tail of each individual mouse according to the method of Gross(6). Reaction time was measured with a stopwatch using the heat induced movement of the tail as the end point. The intensity of this pain stimulus was set by means of a rheostat to obtain an average reaction time of approximately 4 seconds under control conditions. No stimulus was applied for a period longer than 10 seconds in order to avoid burning of the tail. Each mouse was stimulated twice during the tests. The 20 values thus obtained were averaged and standard errors calculated. For reasons indicated above animals which did not react in 10 seconds were given a value of 10 in the averages. Some points of the curves, therefore, represent less than true values; these are identified by rings and true values by dots. Usually 2 control reaction times were obtained following which the drugs were injected and further readings made at regular intervals over a period of 3 hrs. The effects of morphine sulfate, reserpine, and chloropromazine, injected subcutaneously were determined in various doses.

Results. In confirmation of the work of Bein, Gross, Tripod and Meier(5), we observed that reserpine in doses up to 10 mg/kg subcutaneously did not alter the pain threshold in mice. Morphine in doses of 10 mg/kg subcutaneously (Fig. 1) increased the reaction time markedly in all experiments. In view of the established fact that the onset of reserpine's action is slow even after parenteral administration, this drug was injected 2 hrs previous to the injection of morphine to obtain maximal effects simultaneously. Under those conditions the analgetic effect of morphine was reduced in proportion to the dose of reserpine injected. For reasons of simplicity the effect of only one pair of doses are entered in Fig. 1.

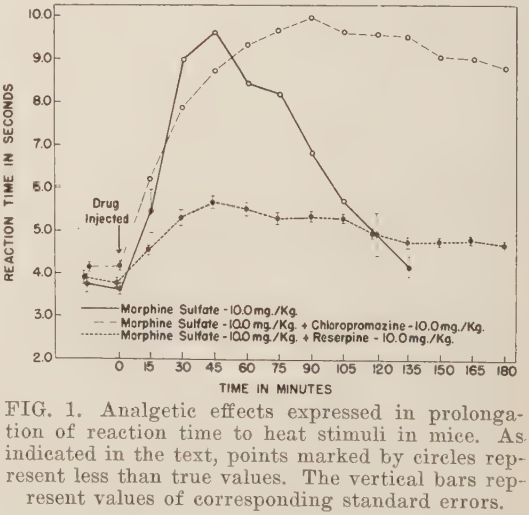

FIG. 1. Analgetic effects expressed in prolongation of reaction time to heat stimuli in mice. As indicated in the text, points marked by circles represent less than true values. The vertical bars represent values of corresponding standard errors.

Although chloropromazine has been reported to have a tranquilizing effect similar to reserpine in patients(7) it differs from reserpine in that it produced a slight analgetic effect in a dose of 10 mg/kg. When this dose of chloropromazine was administered in combination with 10 mg/kg of morphine the analgetic effect of the latter was definitely prolonged (Fig. 1).

Discussion. These findings presented above are of importance because they indicate that in medical use reserpine may weaken the analgetic effect of morphine. It will be of interest to determine if such occurs in man also. Chloropromazine has recently been shown to enhance the analgetic effect of narcotics clinically(8), a fact which is well in line with the experimental data in mice. The antagonistic effect of reserpine on morphine is also of particular interest in connection with some more recent observations of Carey(9) who reported most satisfactory results with reserpine on withdrawal symptoms. The available data give no clue as to the mechanism or localization of the reserpine-morphine antagonism or the chloropromazine-morphine synergism. In case of the reserpine-morphine relationship the possibility of a competitive action at various sites of the central nervous system may be considered in view of the fact that other antagonistic effects of reserpine against morphine on pupil diameter and the Straub tail phenomenon in mice were previously reported by Tripod, Bein, and Meier(10). More recently, Chen and coworkers(11) found that reserpine antagonizes the anticonvulsant effect of Dilantin. Whether the same mechanism of action is involved in these different findings remains to be seen.

The results presented in this paper differentiate further the mode of action of two drugs, reserpine and chloropromazine, which seem to have a remarkably similar action on the central nervous system from a clinical point of view.

Summary. Reserpine (Serpasil) antagonized the analgetic effect of morphine in mice whereas chloropromazine prolonged it.

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Decline of Corticosteroid Secretion Following Hypophysectomy.[*][†] (21462)

Max L. Sweat and Gordon L. Farrell. (Introduced by George Sayers.)
From the Department of Physiology, Western Reserve University School of Medicine, Cleveland, O.

That the pituitary regulates the steroid secretion of the adrenal gland is generally accepted on the basis of indirect evidence. In the present report, experiments are described in which pituitary adrenal relationships have been studied in the dog by a direct approach, namely, the analysis of corticosterone and 17-hydroxycorticosterone in adrenal venous blood. The accepted concepts of pituitary-adrenal relationships are confirmed and in addition certain time relations concerning the rate of adrenal secretion under the influence of the pituitary hormone are demonstrated.

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[†] A preliminary report was read at the Thirty-sixth Annual Meeting of the Endocrine Society (San Francisco, June 17-19, 1954).

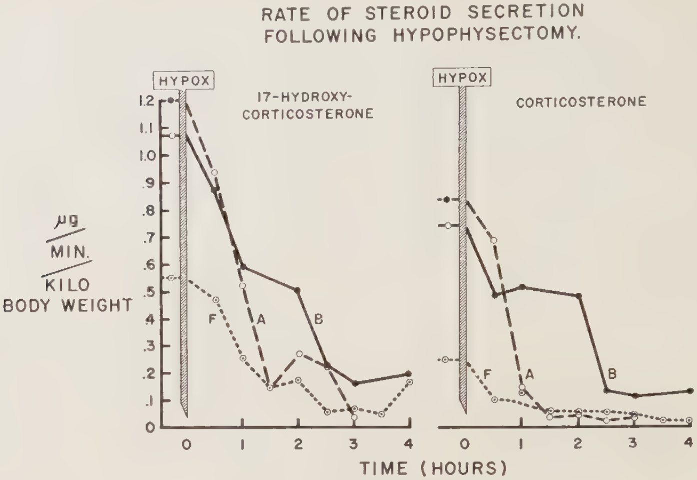

FIG. 1.

Methods. Male mongrel dogs were used in this study. Sodium pentobarbital was employed as anesthetic. At the beginning of each experiment the pituitary was exposed via the oral approach. Care was taken not to injure the gland. The left lumboadrenal vein was then cannulated for collection of adrenal venous blood; the adrenal vein was ligated. Three to 4 mg/kg heparin were injected intravenously at 2-hour intervals. Three consecutive 15 or 30 minute control collections of adrenal venous blood were obtained, after which the pituitary was quickly removed by suction following incision of the dura. From 8 to 12 consecutive 15 to 30 minute collections of adrenal venous blood were then obtained. During the collection periods a mixture of whole blood and 0.9% NaCl (1:1) was infused at a rate approximately equal to the rate of bleeding from the adrenal vein. The blood used for transfusion was obtained from donor dogs and kept at room temperature for 1 to 4 hours to induce destruction of ACTH. Three sham operations were conducted in which the operative procedures were essentially identical to those described above, with the exception that the pituitary was not removed. One to 4 ml aliquots of each sample of adrenal venous blood were extracted with chloroform, partitioned between 70% ethanol and petroleum ether and chromatographed on silica gel(1). The effluents from the chromatography were evaporated to dryness and 0.5 ml of absolute ethanol were added to the residue. One-tenth ml aliquots of the ethanol solutions were used for fluorescent analysis(2).

Results. The rates of secretion of corticosterone and 17-hydroxycorticosterone by the adrenal glands of the 6 dogs are shown in Fig. 1 and 2. It will be noted that a distinct drop in the rate of secretion of 17-hydroxycorticosterone occurs 30 minutes after hypophysectomy; at the end of one hour the rate of secretion of this steroid is approximately one-half that of the pre-hypophysectomy controls. At the end of 3 hours the rate of secretion is one-sixth to one-tenth the pre-hypophysectomy levels. In general, the rate of decline of secretion of corticosterone is equal to that of 17-hydroxycorticosterone. The plateau observed in the secretion of corticosterone for one animal is attributed to individual variation. The secretion of steroids in the sham experiments remains at high but somewhat variable levels (Fig. 2). These data indicate that rapid changes in the rate of steroid secretion may occur in the intact animal. It is noteworthy that for short periods of time the rate of steroid secretion may fall at rates comparable to those observed in the hypophysectomized animals. The essential difference in the two groups of animals appears to be that after an initial drop, the rates of steroid secretion in the intact animals return to high levels of secretion or level off at points significantly higher than the levels of hypophysectomized

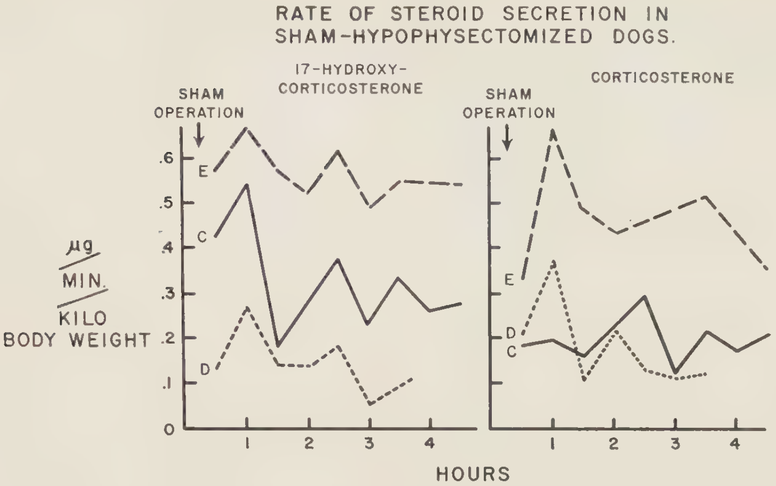

FIG. 2.

animals. However, the rate of steroid secretion in dog D is relatively low and shows a gradual downward trend. At present there is no explanation for the results obtained in dog D. Only a small number of animals have been studied and these have varied in regard to breed, age, weight and state of health. As blood pressure was maintained by transfusions in these animals, the decline does not appear to be due to loss of blood.

In general, rates of decline in the secretion of 17-hydroxycorticosterone by the 3 hypophysectomized animals appear to be similar between the time of hypophysectomy and three hours post-hypophysectomy. After three hours the rate of secretion appears to remain relatively constant at a low level. The persistence of adrenal secretion for a considerable period of time after hypophysectomy raises an interesting speculation. This phenomenon may be due to 1) the stimulating influence of small quantities of ACTH which remain in the circulation following removal of the hypophyses or 2) the maintenance of synthetic activity following the loss of the hormone which stimulates this activity. These experiments do not permit a definitive statement as to which of these alternatives is the correct interpretation. However, the experiments of Sydnor and Sayers(3) which indicate

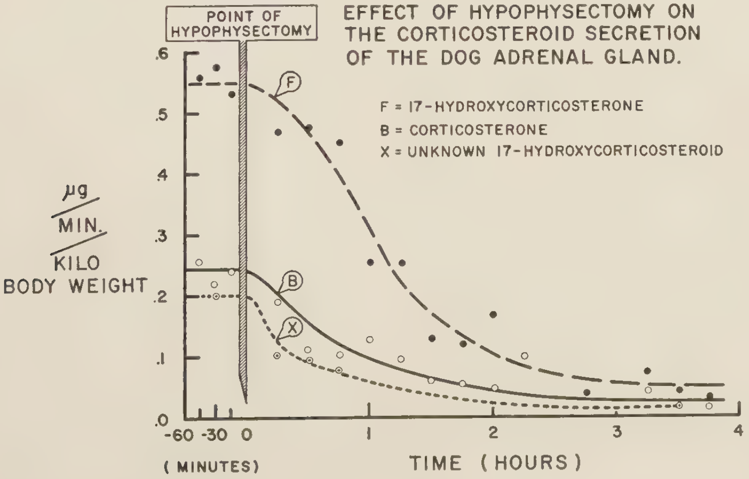

FIG. 3.

that the half-life of endogenous ACTH is one minute strongly suggests that the second possibility is the more likely.

The rate of secretion of a third substance was followed in one animal before and after hypophysectomy (Fig. 3). This substance was quantitated by means of the phenylhydrazine reagent with which it formed a derivative having an absorption maxima in the 400 mμ region. When chromatographed on the silica gel column, the substance was eluted in the same fractions as Substance "S". However, whether this is the same substance tentatively identified as Substance "S" by Farrell and Lamus(4) in their paper chromatography studies on dog adrenal venous blood has not been established. The rate of secretion of this substance after hypophysectomy declines rapidly to low levels of secretion. The rate of secretion (17-hydroxycorticosterone as a standard) was 2.67 μg/minute/kg in the prehypophysectomy controls; 0.46 μg/minute/kg after 4 hours.

The variable rate of steroid secretion in the sham operated animals deserves comment. It will be noted (Fig. 2) that during certain periods of time the rates of steroid secretion may decrease at rates comparable to those observed in the hypophysectomized animals. At other times there was a rapid increase. The observation suggests that rate of discharge of pituitary ACTH underwent marked variations during the experimental period.

The results of these experiments are in accord with those of De Gurpide(5) who has also observed a decrease of 17-hydroxycorticosterone in the adrenal blood of dogs following hypophysectomy. It appears that a similar decline in the concentration of corticosteroids occurs in the perfusates of extirpated beef adrenal glands after the withdrawal of ACTH from the perfusion medium(6).

Summary. The rates of secretion of 17-hydroxycorticosterone and corticosterone prior to and following hypophysectomy have been determined. The rates of secretion of these steroids decline after hypophysectomy, reaching a low and apparently constant level after 3 hours.

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Actively and Passively Induced Arthus Reactions in the Mouse.* (21463)

ALBERT A. BENEDICT AND ROBERT L. TIPS.† (Introduced by Morris Pollard.)
From Department of Preventive Medicine and Public Health, University of Texas Medical Branch, Galveston, Texas.

By employing quantitative methods of antibody determination it has been shown that severity of Arthus reaction in the rabbit(1), guinea pig(2) and rat(3) is related to concentration of circulating antibody, and that this phenomenon may differ fundamentally from anaphylaxis. Direct and reverse passive sensitization procedures were used in these investigations. Although there have been a considerable number of studies dealing with anaphylaxis in the mouse, we have failed to find reports concerned with the Arthus phenomenon in this animal. It was the purpose of the present study therefore to investigate the conditions necessary to produce the Arthus reaction in mice, applying both active and passive sensitization technics.

Materials and methods. Active sensitiza-

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† Department of Bacteriology.

tion. Male and female white mice, strain CFW, weighing 10-15 g were used. Mice received 3 intraperitoneal injections (4 days apart) of 0.1 ml of 0.1-10 mg crystallized bovine plasma albumin (BPA) (Armour) in Freund's adjuvant(4). Equal volumes of BPA solution and adjuvant were emulsified; the adjuvant consisted of Bayol F, Arlacel A (9:1) and 6-10 mg/ml of lyophilized heat-killed tubercle bacilli. *Passive sensitization.* White female mice, strain CF1 (Carworth Farms), weighing 15-20 g, were also used in these studies. Mice which had been actively sensitized with BPA and shown to have a positive Arthus reaction, were bled by heart puncture and the sera were pooled. This pool contained 0.60 mg antibody nitrogen (AbN) per ml, as determined by the quantitative precipitin method(5). A rabbit antiovalbumin serum‡ also used for passive transfer studies, contained 0.20 mg AbN per ml. Reverse passive Arthus reactions were demonstrated in the mice by intravenous (IV) inoculation of saline dilutions of BPA or twice recrystallized egg albumin (Ea) in a volume of 0.5 ml followed by intradermal inoculation of antibody at varying intervals of time. Reverse passive Arthus reactions were demonstrated in guinea pigs using the production and grading technics described by Benacerraf and Kabat(2). *Intradermal inoculation and grading of reactions.* The flanks of the mice were shaved with electric clippers and a razor. Occasional nonspecific irritation which resulted from shaving did not interfere with the reading of the tests. Intradermal injections were performed by passing a No. 27 needle through the skin, reversing its direction, and depositing 0.05 or 0.1 ml of the inoculum into the dermal layers. Nonspecific reactions were not observed with any of the skin test reagents when tested in nonsensitized control mice. Extents of reactions were judged on the basis of the intensity and diameter of the hemorrhage, 5 and 24 hours following intradermal injection. The reactions were graded as follows: 4 + (10-15 mm, confluent hemorrhage and necrosis), 3 + (10-15 mm petechial hemorrhage, no necrosis), 2+ (5-10 mm, petechial hemorrhage), + (1-5 mm, petechial hemorrhage), ± (central blanched area surrounded by a petechial ring, 3-10 mm in diameter), 0 (no reaction).

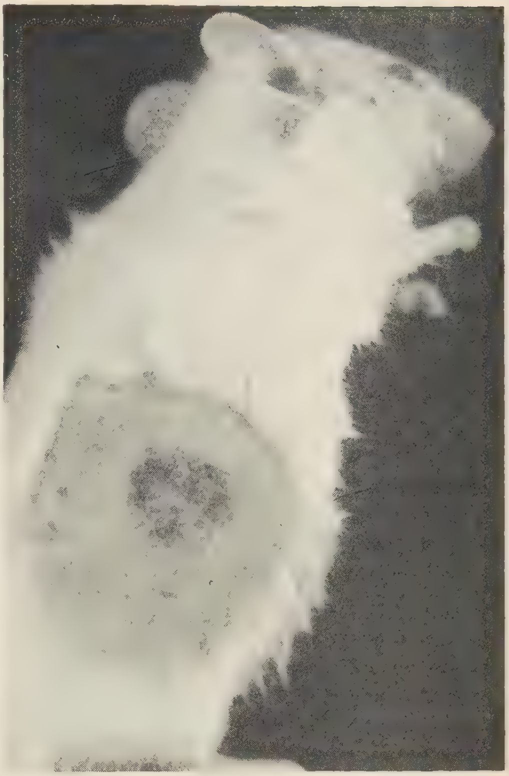

FIG. 1. Twenty-four hr reaction of an actively induced Arthus reaction in the mouse.

Results. The reactions were predominantly hemorrhagic manifesting little or no edema (Fig. 1). In the most severe cases the reaction appeared approximately 2 hours following intradermal injection and was maximal 12-24 hours later. Necrosis invariably occurred when the reaction showed confluent hemorrhage.

Table I summarizes the results of the Arthus reactions produced in mice actively sensitized to BPA. Arthus reactions were not induced 8 days following the last sensitization inoculation. Mice which had received either single or repeated skin tests showed Arthus reactions of various intensities 35 days following sensitization. Table I also shows that 0.1 mg BPA in adjuvant sensitized 50% of a small group of mice tested. Three intraperitoneal injec-

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‡ Generously provided by Dr. Myron S. Silverman, Biological and Medical Sciences Division, U. S. Naval Radiological Defense Laboratory, San Francisco, Calif.

TABLE I. Relationship between Sensitization Period and Severity of the Arthus Reaction in Mice Actively Sensitized to Bovine Plasma Albumin, 7 Groups.

Sensitizing dose, mg BPA	Intradermal test, days after last sensitizing dose	Reactions 4+	3+	2+	+	±	0	% positive
5*	8†						21‡	0
	13					3	16	16
	35	6	3	4	4		2	90
5*	8						10	0
	21					1	8	12
	35	2	1	3		1	1	87
5	26			1	1	2	1	80
10	35	6		2	1	2		100
5	35	9	3	5	3	6	2	93
1	35	1	2		1	1		100
.1	35	1	1	1			3	50

* Groups skin tested 3 times on days indicated; remaining groups received single intradermal inoculations.

† Inoculated intradermally with 0.5 mg BPA; 1 mg BPA used for all other tests.

‡ No. of mice showing reaction.

tions of 5 mg each of BPA in adjuvant followed 35 days later with intradermal test of 1 mg BPA was adopted as the standard procedure for developing Arthus sensitivity.

Since anaphylactic sensitivity has been demonstrated to be greater in the female than the male mouse(6), studies were conducted for a similar comparison of Arthus reactivity. There was no apparent difference between the sexes.

Hemophilus pertussis has been shown to enhance anaphylactic sensitivity to proteins in the mouse(7). To determine whether these organisms enhance Arthus reactivity, 15 mice were given a single intraperitoneal injection of 10 mg BPA incorporated in 0.1 ml of suspension containing 2.5 x 10^9 pertussis organisms.§ Another group of 12 mice received a similar inoculation with the BPA and pertussis cells suspended in an equal volume of adjuvant. In no case was an Arthus reaction noted, when the animals were tested intradermally with 5 mg BPA 7 and 11 days following inoculation. It was surprising to find that 6 of the 15 mice (sensitized by pertussis and BPA) died of anaphylactic shock immediately following the skin test, since Malkiel *et al.*(7) reported that BPA was a relatively poor antigen for anaphylaxis even when the shocking dose was given intravenously. Anaphylaxis was not observed in the group which received BPA and pertussis cells in adjuvant.

Passive sensitization. The results in Table II show that mice inoculated IV 30 minutes previously with 0.15 mg BPA N showed minimal reactions to the intradermal injection of 0.06 mg mouse-BPA AbN, whereas 0.005 mg AbN sufficed to elicit minimal reactions in guinea pigs. Arthus reactions were not induced with the Ea-rabbit antiovalbumin system in either CFW or CF1 mice by the following procedures: 1) IV inoculation of 4.1, 0.15, 0.04 or 0.02 mg EaN followed either immediately, 30 minutes or 24 hours later by intradermal inoculation of 0.02 or 0.01 AbN, 2)

TABLE II. Reverse Arthus Reaction in Mouse and Guinea Pig in Relation to Amount of Mouse Anti-Bovine Plasma Albumin.

Antibody inj. intraderm., mg N	Reactions: Mice 2+	Mice +	Mice ±	Mice 0	Guinea pigs 3+	Guinea pigs 2+	Guinea pigs +	Guinea pigs ±	Guinea pigs 0
.06	1*	4	3	3	1	1			
.03				8		2			
.02				5			4		
.01	(—)						2	1	1
.005	(—)							2	2

Animals inoculated with 0.15 mg BPA N intravenously 30 min. before antibody.

* No. of animals showing reaction.

(—) Not tested.

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§ Pertussis Bacterin (Sharp & Dohme) kindly supplied by Dr. Harriet Felton, Department of Pediatrics, University of Texas Medical Branch, Galveston.

TABLE III. Reverse Arthus Reaction in Mouse and Guinea Pig in Relation to Amount of Rabbit Antiovalbumin.

Antibody inj. intra-derm., mg N	Reactions							
	Mice				Guinea pigs			
	2+	+	±	0	2+	+	±	0
.02				10*	1	1	1	
.01				7		1	1	1
.005	(—)						1	2

Animals inoculated with 0.15 mg EaN intravenously 30 min. before antibody.

* No. of animals showing reaction.

(—) Not tested.

IV inoculation of 0.2 or 0.1 mg AbN followed within 30 minutes by 0.02 mg EaN intradermally, 3) intradermal inoculation of 0.02 mg AbN followed 30 minutes later by inoculation into the same site with 0.02 EaN. Since it was not possible to use larger antibody concentrations with the rabbit serum presently available, the above quantities of antibody were tested for reactivity in guinea pigs. The results summarized in Table III indicate that in order to produce reverse Arthus reactions of minimal severity in the guinea pig, amounts of antibody of the same magnitude as that reported by Benacerraf and Kabat(2) are required. Whether the mouse would be susceptible to sensitization by larger quantities of rabbit antibody than those employed here remains an open question.

Discussion. The guinea pig, which is a poor precipitin producer, has not been considered a suitable animal for consistent demonstration of active Arthus sensitization. Kenton(8) was unable to produce the reaction in the rat by active sensitization. There is sufficient evidence, however, to show that when proper amounts of antibody are injected a passive Arthus phenomenon can be induced in both these species(2,3). The use of Freund's adjuvant(4) for enhancing antibody response is well recognized, and by its use, active Arthus sensitization was consistently demonstrated in the mouse. In addition, mouse sera with quantities of antibody adequate for quantitative precipitin analysis were secured. It is felt therefore that under the conditions noted the mouse can be considered a suitable test animal for Arthus studies.

It was apparent from these studies that a larger amount of rabbit ovalbumin antibody would be required for transferring Arthus reactivity to the mouse than for production of the reaction in the guinea pig. The actual amount required was not determined. To produce minimal reactions with the Ea-anti-Ea system, the rabbit(1), guinea pig(2) and rat (3) have been reported to require 0.025, 0.01 and 0.005 mg of rabbit AbN, respectively.

Generally, passive transfer of anaphylactic sensitivity in the mouse has not been easily attained, even though sera containing 0.6 - 2.0 mg rabbit anti-BPA N have been used (7,9). Malkiel *et al.*(7) were not able to detect precipitable or univalent antibody in mice in which anaphylaxis was enhanced with pertussis organisms, and Weiser *et al.*(10) reported that anaphylactic sensitivity remained after circulating antibody had disappeared. Fink and Rothlauf(11) suggested that anaphylaxis in the mouse was not dependent on circulating antibody. It was shown in the present study that passive Arthus reactions were induced in the mouse when a sufficient amount of homologous antibody was used. The level of 0.06 - 0.03 mg BPA AbN required for this was higher than the amount necessary to produce minimal reactions in the guinea pig (0.005 mg AbN). These findings would further substantiate the differences between anaphylactic and Arthus sensitivities.

Summary. 1. Arthus reactions were consistently produced in mice 35 days following sensitization by 3 intraperitoneal injections of BPA in an adjuvant. 2. Male and female mice were equally Arthus-susceptible. 3. *Hemophilus pertussis* organisms did not enhance Arthus sensitivity under the same conditions in which anaphylactic sensitivity is enhanced. 4. Reverse passive Arthus reactions were not detected in mice injected with 0.02 mg rabbit anti-EaN, whereas minimal reactions were produced in guinea pigs with 0.005 mg anti-EaN. 5. Reverse Arthus reactions of minimal severity were produced with 0.06 and 0.005 mg mouse anti-BPA N in mice and guinea pigs, respectively.

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Synthesis of Mucosa and Lymph Phospholipide During Fat Absorption.* (21464)

Raymond Reiser and Julius W. Dieckert.

From the Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station, Texas A. and M. College System, College Station.

The hypothesis that phospholipides may be intermediate in triglyceride resynthesis has been an attractive one since Sinclair observed that the phospholipides of the intestinal mucosa incorporate ingested fatty acids during absorption(1). There has been evidence, however, that although fatty acids are incorporated, and that there is an increased rate of P^{32} turnover in mucosa phospholipides during fat absorption(2,3) the rate of turnover may not be adequate to account for the amount of fat being absorbed(4).

The present study is an effort to solve this problem by comparison of the relative activities of labeled glycerol and fatty acid in ingested triglycerides to the relative activities of the two moieties in the triglycerides and phospholipides of the mucosa and lymph. The results show that the mucosa and lymph phospholipide glycerol have a lower relative specific activity and, therefore, are probably not triglyceride precursors.

Experimental. Mucosa Phospholipides. Three experiments were performed with 3 different analytical procedures and 2 different test fats. In all 3, the rats were trained to eat a fat-free ration at 8 A. M. On the morning of the test, 100 mg of the test fat was mixed with ½ g of the fat-free basal ration. One-half hour later another ½ g of the basal ration was given. The animals were sacrificed 3 hours after the first meal was offered. They all ate the test meals within a few minutes. In the first test, some of the conjugated trilinolein previously used in fat absorption studies(5) was used. The entire small intestine, after careful washing and removal of mesenteric fat, was diced and extracted with alcohol-ether. The lipide was reextracted with petroleum ether and the phospholipides precipitated with acetone. The phospholipides were then saponified in N KOH in alcohol and the percentage of conjugated linoleic acid in the recovered acids determined spectrophotometrically(5). The acidified saponification liquor was oxidized with 0.1 N KIO_4 in 1.5 N H_2HO_4 at room temperature for one hour, the formaldehyde recovered as methylene bismethone and its radioactivity counted as previously described (5). In the second experiment, tripalmitin, labeled with C^{14} in the α position of the glycerol and in the carboxyl of the acid, was used. The analytical procedures were the same as in the first except that the fatty acid activity was measured by direct counting technic.

Several objections have been raised to the analytical procedure used in the above 2 experiments. It has been reported(6) that mucosa phospholipide contains considerable

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quantities of sphingomyelin, which, because of mutual solubility effects, may dissolve in petroleum ether with the rest of the phospholipide. If this is true, sphingomyelin fatty acids would be added to the phospholipide acids to be counted without the compensating addition of glycerol. A further objection was that the nitrogen bases, serine and ethanolamine, from phospholipide saponification, as well as the glycerol phosphate, may produce formaldehyde upon oxidation with KIO_4. To answer these objections, and to analyze the mucosa independently of the intestinal musculature, the following procedure was devised and followed: Six rats were trained and given test meal as described. Small intestines were removed under ether anesthesia and immediately washed with physiological saline. The mucosa was then squeezed out of the intestine and frozen on dry ice. (To slow autolysis, the intestines were kept on cracked ice when not being handled.) *Extraction and purification of lipides:* About 6 g of mucosa were obtained from the 6 rats. The lipides were extracted and purified according to Johnson *et al.*(7) except that trichloroacetic acid was washed from the 6:1 petroleum ether-chloroform solution with 0.25 M $MgCl_2$ solution instead of water, according to McKibben(8). *Isolation of Phospholipides.* The phospholipides were separated from the combined lipides after the methods of Borgström(9) and Fillerup and Mead(10), modified as follows: A column of dry silicic acid, 10 g per 30 mg of total lipide, was prepared by washing with 3 column volumes of methanol, anhydrous acetone, anhydrous ether and chloroform in turn. The total lipide was dissolved in one column volume of chloroform and put on the column. The phospholipides were adsorbed on the column, washed with chloroform, and eluted with methanol. The methanol was removed *in vacuo* at 55° C in a slow stream of nitrogen. The phospholipide was redissolved in chloroform, transferred to a tared tube, dried under nitrogen and weighed. There were approximately 400 mg. *Saponification.* Because of the report that mucosa phospholipides contain large quantities of sphingomyelin(8) the mixed phospholipides were homogenized and differentially saponified by the method of Thannhauser(6) in 0.25 N NaHO at 37°C for 6 days. After decomposition of the soaps at mild acid pH (methyl red), the mixture was chilled in the refrigerator and filtered through a celite pad on a chilled sintered glass filter and washed with water. *Separation of fatty acids of glycerol containing phospholipides.* The fatty acids and sphingomyelin, plus any other unhydrolyzed phospholipides, were dissolved in chloroform, dried with anhydrous sodium sulfate and filtered. The chloroform solution, after concentration to one column volume, was placed on a silicic acid column prepared as described above for the separation of phospholipide, and unhydrolyzed phospholipide removed as before. The chloroform solution of fatty acids was concentrated, filtered, dried and weighed. There were 132 mg. *Purification of glycerophosphate.* The acidified saponification liquor, after removal of the fatty acids, was freed of ethanolamine and serine by passage through a 10 g column of Dowex 50 (acid form) in a 0.50 ml burette, prepared by washing with demineralized water. *Preparation of methylene bismethone.* The purified glycerophosphate solution was diluted to 50 ml with distilled water, and 50 ml of 4 N H_2SO_4 and 36 ml of 0.1 N KIO_4 (equivalent to 0.8 mmole of glycerol) added. The mixture was heated for one hour at 95°-100°C while nitrogen was passed through the solution to flush the resulting formaldehyde into a trap containing 60 ml of 0.4% aqueous dimedone, 40 ml of water, 10 ml of AcONa and acid to pH 4.7 - 5.0. Preliminary studies have shown that under these conditions choline produces no formaldehyde and that one mole of glycerophosphate produces one mole of formaldehyde.

Mucosa "Neutral Fat." The chloroform solution, after removal of phospholipides on silicic acid as described above, was evaporated, and the "fat" saponified with 4% alcoholic KOH. The fatty acids were separated and plated as usual. The acidified saponification liquor was cleared by filtration through Supercel and oxidized with 0.1 N KIO_4 in 1.5 N H_2SO_4 for one hour at room temperature,

TABLE I. Specific Activity of Glycerol and Fatty Acids in Ingested Fats and in Lymph and Mucosa Lipides.

Rat No.	Glycerol			Fatty acids		
	Fat fed	Lymph*		Fat fed	Lymph*	
		Triglyceride	Phospholipide		Triglyceride	Phospholipide
	μc/g	μc/g	μc/g	diene %	diene %	diene %
1.	89.7	50.3† 25.3‡	19.5	85.8	84.0† 44.9‡	39.2
2.	89.7	33.9		85.8	50.1	
3.	17.9	7.7	2.8	17.2	10.2	6.0
4.	44.9	18.2		42.9	29.4	
		Mucosa			Mucosa	
	cpm	cpm	cpm	diene %		diene %
2a.	234,498		10,491	61.2		6.5
				cpm	cpm	cpm
9.	28,465		60	47,829		296
10.	21,503	2153	177	37,693	2447	793

* From previous study(5). † Lymph collected 0-12 hr. ‡ Lymph collected 15-28 hr.

and the methylene bismethone prepared in the usual manner. *Lymph Phospholipide and "Neutral Fat".* The specific activities of the glycerol and fatty acids of lymph triglycerides and phospholipides are already available from a previous publication(5). The significance of the data to the problem of the role of phospholipides in fat absorption did not occur to the author at that time. *Determination of radioactivity.* The activities, in counts per minute, were determined by direct count on the fatty acids and methylene bismethone.

Results. It is assumed, in this series of studies(5,11) that any loss in activity of ingested labeled fatty acid in mucosa or lymph lipide fatty acids is due to dilution with endogenous fat. The glycerol in mucosa or lymph lipides should be diluted to the same degree. Any additional reduction in the activity of labeled glycerol must then be due to hydrolysis and replacement of labeled with unlabeled glycerol. In the studies of lymph lipides(5) the ratio of the specific activity of the fatty acid to the specific activity of the glycerol in the fat fed, compared to the same ratio in the lymph lipide, was used as a measure of the percentage of labeled glycerol which was lost by hydrolysis. For the purposes of the present study it was considered desirable to express the per cent loss of activity of glycerol compared to that of the acid as the relative specific activity of the lipide glycerol. If L = the relative specific activity of the lipide glycerol, Af = the activity of fatty acid in the tripalmitin fed, Ar = activity of fatty acid in the tissue lipide, Gf = activity of the glycerol in the tripalmitin fed, and Gr = activity of glycerol in the tissue lipide, then

$$L = 100 - \frac{Af}{Ar} \times \frac{Gr}{Gf} \times 100$$

The factor "L" also expresses the percentage substitution of labeled with unlabeled glycerol.

In Table I are given the specific activities of the glycerol and fatty acids of the fat fed and of the mucosa and lymph lipides. The values for the lymph studies are from a previous publication(5) in which the activities of the fatty acids are in terms of per cent of conjugated dienoic acid. The activities of the glycerol in the lymph studies are in terms of microcuries per gm of glycerol.

In the mucosa studies, labeled tripalmitin was fed and all specific activities are in counts per minute.

The values of the relative specific activities "L" for the three experiments described herein, and the values recalculated from the previous work(5), are summarized in Table II.

Discussion. The difference between the reduced activity of fatty acid and glycerol due to dilution with endogenous fat, and the additional loss of activity of glycerol expressed by

TABLE II. Relative Specific Activity, "L," of Labeled with Unlabeled Glycerol in Intestinal Mucosa and Lymph Lipides after Ingestion of C^{14} Labeled Glycerol as Tripalmitin and as Conjugated Trilinolein.

Lymph			Mucosa		
Exp.	Triglyceride	Phospholipide	Exp.	Triglyceride	Phospholipide
1	42.7* 45.7†	52.4	2a		58.1
2	35.3		9		60.9
3	27.5	55.2	10	–54	65.9
4	40.8				

* Lymph collected during 0-12 hr.
† " " " 13-28 ".

the factor "L" in Table II, may be further explained by a simple example:

If a triglyceride which contained 100 units of activity in both glycerol and fatty acid were fed, and the resultant lymph triglycerides had 50 units of activity in the fatty acid moiety but only 25 units in the glycerol moiety, there would have been a 1:1 dilution with endogenous fat. In addition, there would have been a 50% substitution ("L") of labeled exogenous glycerol with inactive endogenous glycerol, and the relative specific activity of the lymph triglyceride glycerol would be 50.

The negative number representing the replacement of ingested labeled glycerol with endogenous glycerol in mucosa "neutral" fat may be explained on the basis that this fraction contained considerable quantities of unlabeled free fatty acid. This possibility is currently under study.

The reasonably close agreement between the values of 58, 62 and 61 for the relative specific activities of the mucosa phospholipide glycerol obtained by the use of 3 different procedures, demonstrates that all procedures agree and that both the precaution of differential saponification to remove sphingomyelin from the total lipide, and the ion exchange procedure to remove serine and ethanolamine from the acidified saponification liquor, are unnecessary. Evidently the reextraction of the alcohol-ether extract with petroleum ether is adequate for eliminating the sphingomyelin and, under the conditions used, (acid pH and room temperature for one hour), serine and ethanolamine are not oxidized to formaldehyde with KIO_4. Other studies with these pure bases, as well as choline, have also shown that under these conditions formaldehyde is not a product of their oxidation with KIO_4.

The reasonably close agreement between the relative specific activities of the glycerol in mucosa and lymph phospholipides indicates that the two have the same origin.

The 60% substitution of the ingested labeled glycerol with unlabeled glycerol in mucosa and lymph phospholipide, but only 40% substitution in lymph triglyceride, demonstrates that triglyceride is the precursor of phospholipide rather than the converse.

These observations may be interpreted by the following scheme in which *G* represents labeled glycerol, *A* labeled fatty acid, *gp* unlabeled endogenous glycerol or its precursor, and *PB* a phosphorylated base.

Digestion: $5\ GA_3 \rightarrow 5\ GA + 10A$

Triglyceride resynthesis: $5\ GA + 10A + 2\ gp \rightarrow 3\ GA_3 + 2G$

Phospholipide synthesis: $2\ GA_3 + gp\ PB \rightarrow 3\ GA_2PB$

According to this hypothesis monoglycerides are the end product of fat digestion(11). The absorbed monoglycerides and glycerol, or glycerol precursor (possibly dihydroxyacetone (11)), compete in the mucosa for the available fatty acids in the ratio of 3:2. There is thus a 40 per cent dilution of the original glycerol. Each two moles of the resynthesized triglyceride then form 3 moles of mucosa and lymph phospholipide, resulting in an additional 20% loss in the specific activity of the glycerol as compared to the fatty acid.

Summary and conclusions. Comparison of the relative specific activities of the glycerol of lymph triglycerides and phospholipides from previous studies, and of intestinal mucosa phospholipides from new studies demonstrated that resynthesized triglycerides must be the precursors of mucosa and lymph phospholipides, rather than the converse.

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Effects of Thyroxine and KSCN on Capacity of Rat Thyroid Gland to Accumulate Astatine^{211}.* (21465)

C. J. SHELLABARGER,[†] PATRICIA W. DURBIN, MARSHALL W. PARROTT, AND JOSEPH G. HAMILTON.

From the Division of Medical Physics, Experimental Radiology and Medicine, Crocker Laboratory, University of California, Berkeley and San Francisco.

The discovery and production of Element 85, astatine^{211}(1) was closely followed by studies of the thyroidal accumulation of this, the heaviest of the halogen elements(2). It has been clearly established that the thyroid glands of rats, guinea pigs, monkeys, and man selectively concentrate the 7.3 hour isotope, At^{211}, to a degree which is similar to, but lower than that of I^{131}(2-6). It has been further established that the thyroid stimulating hormone (TSH) increases while iodide decreases the thyroidal accumulation of both At^{211} and I^{131}(2,3). Two recent reports indicate, however, that the thiouracil type of anti-thyroid compound enhances the thyroidal uptake of At^{211} in rats in contrast to lowering the I^{131} uptake(4,6). Since the mechanism of the thiouracil induced enhancement of At^{211} uptake has not been satisfactorily understood or explained, it seemed of interest to study the effects of other classes of anti-thyroid compounds upon the capacity of the thyroid gland to accumulate At^{211}. It is the purpose of this paper to present data which suggest that depressed thyroid function, as produced by the administration of exogenous thyroxine, results in a lowered thyroidal accumulation of both At^{211} and I^{131}, that thiocyanate depresses the thyroidal accumulation of At^{211} and that thiocyanate apparently discharges At^{211} from the thyroid gland.

Methods. The animals used in these studies were young female Sprague-Dawley rats that had been maintained for at least 2 weeks after receipt from the dealer on tap water and a pelleted stock diet[‡] which is in general use throughout the University of California Radiation Laboratory. Both food and water were given *ad lib.* Food was withdrawn following the intravenous administration of either At^{211} or I^{131}.[§] The At^{211} was prepared by a modification of the method described by Garrison *et al.*(7). Following pretreatment with either thyroxine or KSCN and the administration of the isotopes, the animals were sacrificed with chloroform at intervals of from 1 to 18 hours. The thyroid glands were dissected and weighed to the nearest 0.2 mg on a torsion balance. The x-ray activity of the At^{211} or the γ-ray activity of the I^{131} were measured with an NaI-TlI scintillating crystal gamma count-

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[*] This work was performed under contract of University of California with U. S. Atomic Energy Commission.

[†] Present address, Brookhaven National Laboratory, Upton, L. I., N. Y.

[‡] This diet is similar in composition to "Diet 14" developed by the University of California Institute of Experimental Biology and has been found to contain 2.5 μg of iodine per gram.

[§] Processed, carrier-free I^{131} was obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.

TABLE I. Effect of 8 Daily Subcutaneous Injections of Thyroxine (230 μg/kg/Day) on 18 Hour Thyroidal Uptake of I^{131} or At^{211}, Body Weight and Thyroid Weight of 55-Day-Old Female Sprague-Dawley Rats. Last thyroxine injection given 4 hr before IV administration of isotope.

Treatment	No. of rats	Mean body wt beginning, g	Mean body wt at sacrifice, g	Mean thyroid wt, mg	Mean ± S.E. thyroid uptake, % of admin. isotope	Mean ± S.E. thyroid conc., % isotope/g wet tissue
Thyroxine + I^{131}	8	121	133	9.8	*.73* ± .06	*75.4* ± 6.6
Control + I^{131}	10	124	148	12.3	6.00 ± .51	502 ±46
Thyroxine + At^{211}	10	114	124	9.6	*.068* ± .001	*6.9* ± .5
Control + At^{211}	9	119	141	9.7	.305 ± .01	32.0 ± 4.3

Italicized mean value indicates that it was tested against the mean immediately below by the t-test(12) and the P value was beyond the 1% level of confidence.

er which has been described elsewhere(3). Thyroxine was given subcutaneously in a series of 8 daily injections at a level of 230 mg/kg/injection in a volume of 0.1 ml of isotonic saline.‖ The last injection of thyroxine was given 4 hours before the administration of the radiohalogens. An equal number of rats received daily injections of a similar volume of isotonic saline and served as controls. One-half of the thyroxine treated group received 30 μc of At^{211} and the other half, 10 μc of I^{131}. The sham injected control group was divided and received the same amounts of At^{211} or I^{131} as above. The number of animals in each group is shown in Table I. Potassium thiocyanate was given subcutaneously at a level of 80 mg/kg/injection in a volume of 0.2 ml. Each rat received 20 μc of At^{211}. The number of rats in each group, the time schedule and the number of KSCN injections and the interval between the administration of the At^{211} and the sacrifice of the animals is shown in Table II.

Results. Thyroxine. The results of 8 daily subcutaneous injections of thyroxine (230 μg/kg/day) on the 18 hour thyroidal uptake of I^{131} or At^{211}, body weight, and thyroid weight are presented in Table I. The thyroxine dosage was sufficient to depress the growth rate as measured by body weight to a statistically significant degree ($P<0.01$), but did not appear to affect thyroid weight. The thyroxine dosage depressed the thyroidal accumulation of both I^{131} and At^{211} below that of non-thyroxine treated control rats. It should be noted that the thyroid uptake values for both At^{211} and I^{131} are somewhat low. This is presumably due to the presence of a relatively large amount of stable iodine in the diet employed.

KSCN. Table II shows the accumulation of At^{211} by the thyroid gland of the rat 1, 4, and 18 hours following its intravenous injection with and without the administration of KSCN. A subcutaneous injection of 20 mg of KSCN 1.5 hours before At^{211} injection when the rats were sacrificed at either 1 or 4 hours after the administration of At^{211} resulted in a lower thyroidal accumulation of At^{211} as compared to the controls. Rats that received 2 KSCN injections, 1.5 hours before the At^{211} was given and another KSCN injection 14 hours later with an interval between At^{211} administration and sacrifice of 18 hours, had lower thyroid At^{211} values than the controls. When the interval between the administration of At^{211} and sacrifice was 18 hours, a single injection of KSCN 1.5 hours before sacrifice also produced lower thyroid accumulation of At^{211}.

Discussion. It has been clearly established that thyroid hormone acts to depress thyroid function by inhibiting the production of TSH by the anterior pituitary(8). In this experiment, the administration of relatively large amounts of thyroxine apparently inhibited pituitary TSH production which, in turn, depressed thyroid function as measured by the decreased thyroidal accumulation of both At^{211} and I^{131}. These findings are in agreement with the previous studies by Hamilton and Soley(2) that exogenous TSH increased the thyroidal uptake of both At^{211} and I^{131},

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‖ Pure crystalline 1-thyroxine (Squibb), 100 mg dissolved in 182 ml of 0.0035 N NaOH, made isotonic with NaCl and pH was adjusted to 8.5.

TABLE II. Effects of a Single or Double Subcutaneous Injection of KSCN on Thyroidal Uptake of At^{211} in 130-Day-Old Female Sprague-Dawley Rats.

Interval between At^{211} IV inj. and sacrifice, hr	Interval between KSCN inj. and sacrifice, hr	No. of rats	Mean body wt, g	Mean thyroid wt, mg	Mean ± S.E. thyroid uptake, % of admin. At^{211}	Mean ± S.E. thyroid conc., % At^{211}/g wet tissue
1	None	5	252	20.5	.31 ± .02	15.60 ± 1.35
1	2.5	5	254	20.3	*.07* ± .01	*3.19* ± .13
4	None	5	246	16.8	.32 ± .03	18.95 ± 1.36
4	5.5	5	245	16.7	*.10* ± .01	*5.85* ± .45
18	None	6	248	19.9	.40 ± .06	20.00 ± 2.16
18	19.5	6	244	18.8	.45 ± .08	23.08 ± 3.34
18	19.5-5.5	6	255	18.4	*.16* ± .02	*8.67* ± .52
18	1.5	6	248	21.0	*.22* ± .02	*10.37* ± 1.12

Italicized mean value indicates that it was tested against the mean of the control non-KSCN injected group of the same interval between At^{211} injection and sacrifice(12) and the P value was beyond the 1% level of confidence.

and further indicate that the thyroidal accumulation of At^{211} is under pituitary control as is the thyroidal accumulation of I^{131}.

It has been demonstrated that thiocyanate inhibits the collection of iodide by the thyroid gland(9). It seems reasonable to interpret the present finding of a lowered thyroidal uptake of At^{211} when thiocyanate was given prior to At^{211} administration to a similar mechanism, that is, thiocyanate interferes with the capacity of the thyroid gland to concentrate astatide (At^-).

What happens to the astatide after it is collected by the thyroid gland is more difficult to understand. In the case of I^{131}, iodide is presumably oxidized to iodine and this is organically bound(10). Thiouracil blocks the organic binding of iodine, and this is reflected by a lower I^{131} content of the thyroid gland. However, both thiouracil and propyl thiouracil have been shown to increase the accumulation of At^{211} by the thyroid gland(4,6). These results have indicated that the behavior of the thyroid toward At^{211} and I^{131} is not always similar. This is not surprising since the chemistry of astatine and iodine differ in many respects(11). It has been shown that thiocyanate administration after the organic binding of I^{131} discharges little or no I^{131}(9). Since in the present experiment approximately 50% of the At^{211} was discharged when KSCN was given 1.5 hours before an 18 hour At^{211} uptake, it is apparent that the At^{211} accumulated by the thyroid is not organically bound in a manner that is strictly comparable to the organic binding of I^{131}.

Summary. 1. The administration of thyroxine to the intact female Sprague-Dawley rat resulted in a lowered thyroidal accumulation of both At^{211} and I^{131}. Since it is known that thyroxine inhibits production of TSH by the pituitary gland, it is suggested that a reduced pituitary function resulted in a reduced thyroidal accumulation of both halogens. It is further indicated that the thyroidal accumulation of At^{211} is under pituitary control. 2. It has been demonstrated that thiocyanate blocks the thyroidal accumulation of astatide, and that since thiocyanate can discharge At^{211} from the thyroid gland, At^{211} probably is not organically bound in a manner similar to the organic binding of I^{131}.

Authors wish to extend sincere thanks to Mr. G. B. Rossi and the staff of the 60-Inch Cyclotron who were responsible for the production of the astatine, to Miss Margaret Gee, Mrs. Marilyn Williams, Mrs. Ruth Newman, and Miss Nylan Jeung who gave technical assistance and to Miss Catherine Sjoblom who prepared the manuscript.

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Preventive Influence of Certain Amino Acids on Experimental Fatty Liver of Portal Type.* (21466)

MAURICE E. SHILS AND W. B. STEWART.

From the School of Public Health and Department of Pathology, College of Physicians and Surgeons, Columbia University.

Dietary deficiencies or imbalances of various amino acids have caused fatty liver in rats despite the presence of choline in the diet. Lysine and threonine prevent fatty liver which occurs with diets containing mixtures of purified amino acids deficient in the respective substance(1,2); when 9% casein is the source of amino acids threonine is beneficial(2-5). Tryptophan deficiency has been implicated in the development of fatty liver following forced-feeding of diets containing acid-hydrolyzed casein(6). We have reported the rapid and consistent occurrence of a portal type of fatty liver in rats subsisting on diets containing vegetable protein and devoid of protein of animal origin(7). Choline and vit. B_{12} did not prevent the development of this type of fatty liver, while DL-methionine added to the diet maintained normal hepatic lipid only when present at toxic levels(8). The present report summarizes our findings on the effectiveness of certain amino acids in preventing the accumulation of lipid produced by diets containing plant proteins.

Methods. The type of rat and chemical and histological procedures were identical to those reported previously(9). In most instances littermate rats of both sexes were distributed among the dietary groups. The data from both sexes are combined in the tables except for the one experiment where there appeared to be a consistent sex difference. The animals were placed on the diets at weaning (21 days) and killed after 21 or 28 days. The chief ingredients of the 3 diets used were either American white cornmeal, ground white rice, or cassava flour at a level of 74 parts. To each of these were added vitaminized cerelose, hydrogenated vegetable oil, salts, cod liver oil and corn oil as described elsewhere(7). The amino acid supplements were: DL-tryptophan (0.5%) L-lysine HCl (1%), DL-threonine (0.5%) and DL-methionine (0.5%). Lipids are expressed on a wet weight basis.

Results. Tryptophan and lysine, alone and together, greatly reduced or prevented the accumulation of hepatic fat usually seen on the cornmeal diet (Table I). Threonine, on the other hand, had no significant effect on the male rats, but reduced somewhat the fatty liver of the female rats. The addition of 0.5% methionine to the other amino acids seemed to have had little or no additional effect upon the hepatic lipid content. This is in contrast to an earlier finding(8) where methionine alone caused higher lipid values in male rats. None of the supplements had any consistent influence on the rate of growth.

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TABLE I. Influence of Certain Amino Acids on Hepatic Lipid Concentration and Growth of Rats Subsisting on Cornmeal Diet for 28 Days.

	Supplement									
	None	T*	T + M	L	L + M	Th		Th + M	L + T	T + L + M
No. of rats	34	20	13	19	8	10 ♂	8 ♀	15	21	15
% lipid										
Avg	13.5	5.5	6.8	6.8	4.8	11.2	9.7	8.4	5.4	4.9
Range	5.0–20.9	3.1–12.1	3.2–11.3	3.7–14.2	2.7–7.9	5.7–15.6	4.1–15.6	2.4–14.8	2.8–7.9	2.7–9.3
Growth (g)										
Avg	12	7	7	12	8	10	10	9	16	13
Range	5–22	3–16	3–11	4–19	−2 to 16	6–17	6–13	5–12	5–28	2–35
P†		.001	‡	.001	‡	.57	.01	.06	.001	.001

* T = Tryptophan; M = Methionine; L = Lysine; Th = Threonine.

† P = Probability by the "t" test that the mean difference using paired littermates is due to chance. In each instance the rats in a treated group were compared with their littermates on the unsupplemented diet.

‡ Insufficient numbers of littermate rats for statistical analysis.

The preventive effect of tryptophan and lysine when added to the rice diet was very striking (Table II); only one treated rat had a value that could be considered elevated. The addition of threonine or of threonine and methionine to the combination of lysine and tryptophan did not alter the results. Neither lysine nor tryptophan influenced growth, but the addition of threonine to this combination caused a relatively marked weight gain. The amino acids added to the cassava diet were effective, but the differences are not as striking because of the lower lipid values of the unsupplemented group.

Microscopic examination of the livers supported the chemical data obtained on the cornmeal diet (Table III). Only small amounts of fat were seen in the livers of rats which had received tryptophan or lysine, or both. When the fat was visible and localized, it was in the portal areas in all but two livers. About half of the rats listed as having diffuse fat had only small amounts of stainable lipid which was scattered about in the lobules without definite localization. All rats in this category had received lysine, either alone or in combination. Most of the rats fed the rice or cassava diets with added amino acids had little or no stainable fat, and the remainder had small amounts in the periportal areas.

Discussion. The results, in conjunction with the work of others(1-6), emphasize the importance of amino acid deficiencies or imbalances in the production of fatty livers. They also serve to underscore the point made by Harper *et al.*(5) that the effectiveness of a particular amino acid (or amino acids) in preventing fatty liver will depend upon the composition of a given dietary protein and upon the availability of its amino acids to the organism. For example, the relative ineffectiveness of threonine on the cornmeal diet contrasts sharply with its effectiveness on a 9% casein diet(3-5) or on certain diets with purified amino acids(2). With the proteins of corn, rice, and cassava, tryptophan or lysine

TABLE II. Influence of Certain Amino Acids on Hepatic Lipid Concentration and Growth of Rats on the Rice and Cassava Diets for 21 Days.

	Rice diet					Cassava diet				
	No. of	% lipid		Growth (g)		No. of	% lipid		Growth (g)	
Supplement	rats	Avg	Range	Avg	Range	rats	Avg	Range	Avg	Range
None	14	14.7	6.0–20.2	12	10–16	8	6.7	5.4–9.2	−7	−5 to −10
T* + L	8	3.6	2.7– 5.2	8	4–15	4	4.5	3.4–5.3	−8	−6 to −10
L + T + Th	8	4.3	3.2– 5.2	28	21–31	—	—	—	—	—
T + L + Th + M	6	3.6	2.9– 4.3	21	16–39	3	3.5	2.9–4.1	−8	−6 to −10

* T = Tryptophan; L = Lysine; Th = Threonine; M = Methionine.

TABLE III. Microscopic Distribution of Lipid in Hepatic Lobules of Rats Subsisting on Cornmeal Diet with Amino Acid Supplements.

Supplement	No. of rats	Site of lipid in lobule				Very little or no fat
		Portal				
		Definite	Tendence	Central	Diffuse	
T*	15	8†	0	0	2	5
T + M	13	9	0	1	1	2
L	14	3	3	0	5‡	3
L + M	8	2	1	0	3‡	2
Th	16	7	2	0	5	2
Th + M	9	7†	1	0	1	0
T + L	20	11†	1	1	1	6
T + M + L	15	7†	2	0	1‡	5
Total	110	54	10	2	19	25

* T = Tryptophan; M = Methionine; L = Lysine; Th = Threonine.

† Most of the livers in this group had relatively small amounts of stainable fat which was localized just about the portal connective tissues.

‡ Relatively small amounts of stainable fat present which was scattered diffusely throughout the lobule.

or both are key substances in prevention of fatty liver. With corn, however, still other nutrients may be involved since some of the treated rats had liver lipids above normal.

Our findings also suggest that amino acids required for growth are different from those influencing the deposition of fat in the liver. The amino acids that prevented the fatty liver had no consistent effect upon the rate of growth. Furthermore, threonine, which was not necessary to prevent fatty liver on the rice diet, improved growth when added to the combination of lysine and tryptophan. This latter observation recalls the finding of Pecora and Hundley(10) that threonine and lysine together improve growth of rats on rice diets.

Summary. Certain amino acids have been tested for their ability to prevent the portal type of fatty liver which develops in rats subsisting on diets in which the protein was derived from cornmeal, rice, or cassava. DL-tryptophan (0.5%), and L-lysine HCl (1%), individually or together, partially or completely prevented the fatty liver. Threonine had little or no effect. The amino acid requirements for prevention of fatty liver were not correlated with requirements for growth.

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Effect of Cyanide Poisoning on the Central Nervous System of Rats and Dogs.* (21467)

C. L. Rose, P. N. Harris, and K. K. Chen.

From the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

Cyanide poisoning is an acute event ending in either death or prompt recovery. In man successful treatment can be achieved by inhalation of amyl nitrite and intravenous injection of sodium nitrite, followed by sodium thiosulfate(1). To date 48 recoveries are on record. Two cases, both recorded by Williams (2), showed a period of excitement and violence after adequate treatment and the disappearance of toxic signs from hydrocyanic acid. One of Williams' case histories was related in our paper(1). Töppich(3) described the occurrence of capillary congestion and degeneration of Purkinje cells in 2 persons who died about 4 hours after contact with cyanide. Wheatley and his co-workers(4) reported that cats could sustain repeated cyanide convulsions without presenting any demonstrable damage to the central nervous system. In dogs, on the other hand, Jedlowski(5,6) found leukoencephalopathy attributable to demyelinization after repeated injections of potassium cyanide. His work was confirmed by Hurst (7) in monkeys and by Lumsden(8) in rats. A similar observation was recorded by Dechaume and Chambon(9). One of 3 dogs that received daily doses of potassium cyanide died in 190 days, and its brain showed areas of necrosis.

The importance of this problem prompted us to carry out experiments in animals poisoned with potassium or sodium cyanide with or without nitrite-thiosulfate treatment. Our object was to find out whether the cyanide *per se* or its detoxified product, the thiocyanate, was responsible for any deleterious effect on the central nervous system. Further, could the nitrite be injurious to the brain by causing methemoglobinemia? Therefore, sodium nitrite and potassium thiocyanate were studied separately in our experimental animals.

Procedure. Young rats of approximately 100 g were employed. One series of 100 rats, 10 to a cage, received subcutaneously a daily dose of potassium cyanide, 3 mg/kg for the first week. The dose was then increased by 1 mg/kg and held constant as long as deaths occurred, but was increased again when there were no deaths. The final daily dose was 14 mg/kg, and after 118 days only 1 rat survived. One hundred rats of a second series were injected intraperitoneally with sodium nitrite. The initial daily dose of 60 mg/kg had been increased to 109 mg/kg when the experiment ended after 108 days. A third series comprised 75 rats. These animals were given daily intraperitoneal injections of potassium thiocyanate, starting with 50 mg/kg for the first 80 days, and terminating with 850 mg/kg on the 103rd day. Dosage for these 2 groups was increased according to the principle applied to the first group. Three dogs were injected subcutaneously with a daily dose of sodium cyanide, 3 mg/kg. One of the 3 received 4 mg/kg during the last 2 weeks of its life. Six additional dogs were poisoned with multiple lethal doses of sodium cyanide, 60 mg/kg, but promptly treated by intravenous injection of sodium nitrite (25 mg/kg) and sodium thiosulfate (2.5 g/kg). The antidotes were repeated when toxic signs appeared. *Necropsies* were performed on those animals that had not undergone post-mortem changes. Sections of the brain and other organs were routinely stained with hematoxylin and eosin. Two blocks of cerebrum and occasionally one block of cerebellum were taken from each brain. When intracranial lesions were found, special methods for staining myelin were employed.

Results. A. *Rats.* 1. *Potassium Cyanide.* Forty-one rats died during the first week of daily injection. Their brains were not examined. Seven rats were lost to cannibalism during the remaining course of the experiment. This left 52 rats, which eventually died on in-

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* The authors are indebted to Mr. Robert D. Fink for carrying out many of the experiments.

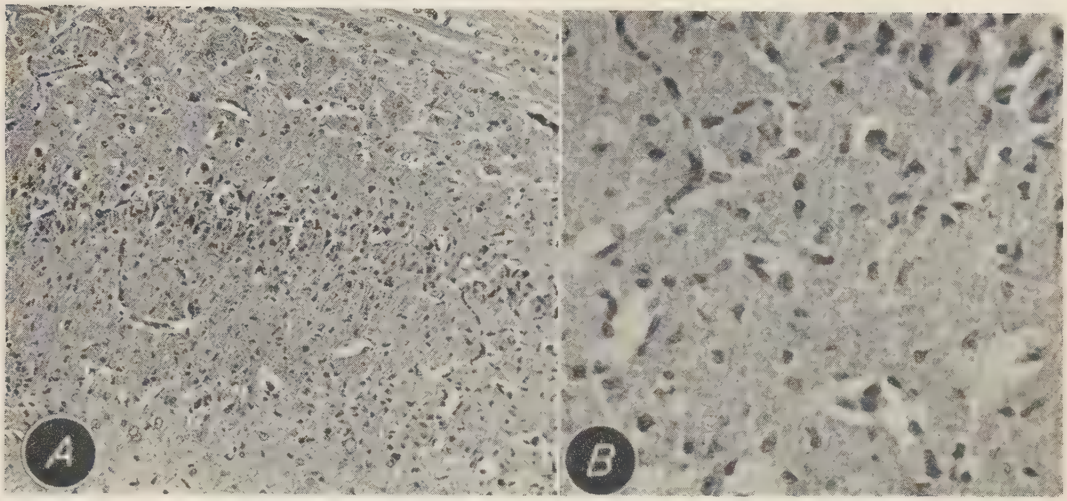

FIG. 1. Cerebral changes in cyanide poisoning. A rat was inj. subcut. with daily doses of potassium cyanide. It died on 28th day. Brain section stained with hematoxylin and eosin. A, 160×. Extending horizontally across the field is a zone of necrosis that involves all large ganglion cells of Ammon's horn. Tissue near the ganglion cells contains moderate numbers of cells that resemble migrating leukocytes. B, 400×. A portion of field in Fig. 1 to show cell types more clearly. Necrotic ganglion cells form a diagonal band across top of the field.

creasing daily doses except 1 that survived 118 days. This animal was sacrificed. Intracranial lesions were found in only 6 of the 52 rats. One that died on the 50th day of cyanide poisoning had bilateral subdural hemorrhage, most extensive posteriorly and laterally; there was no microscopic lesion. The other 5 rats died on the 27th to 107th days of treatment with microscopic lesions involving the corpus callosum or the hippocampus. The rat that died on the 27th day had both callosal and hippocampal lesions, and 1 that died on the 28th day had a hippocampal lesion only. The hippocampal lesion consisted of symmetrical and extensive bilateral necrosis of the large ganglion cells of Ammon's horn. In the necrotic and adjacent tissue were cells with darkly stained, elongated, irregular, or even lobulated nuclei. They suggested wandering leukocytes, but there were no leukocytes in perivascular spaces anywhere in the brain. The lesion is illustrated in Fig. 1A and B. The lesion in the corpus callosum consisted of focal rarefaction and demyelinization with diminution in, or profound alteration of oligodendroglial cells. The number of cells in these foci was increased, and the cells in general were longer and flatter than oligodendroglial cells. The nuclei were darkly stained, with detail indistinct or obliterated. Some nuclei were elongated and resembled those of migrating leukocytes. This type of lesion is shown in Fig. 2. The fourth rat died on the 29th day; it showed a similar lesion of the corpus callosum. The remaining 2 rats died on the 107th day, 1 with a callosal lesion (depicted in Fig. 2), and the other with a small hemorrhage in the corpus callosum.

2. *Sodium Nitrite.* Of 100 rats injected with increasing doses of the nitrite for 108 days, 11 that died were devoured by their cage mates. Eighty-nine were brought to autopsy, and their cerebral tissues were examined microscopically. No significant changes were observed in the brains of any of these animals.

3. *Potassium Thiocyanate.* Out of 75 rats, 36 were lost to cannibalism; the brains of 39 were studied upon death or sacrifice (the last rat on the 103rd day). Brain lesions were present in 3 rats. One rat that died on the 13th day showed a focus of hemorrhage at the anterior end of the corpus callosum (Fig. 3) and 2 other hemorrhagic foci inferior to the hippocampus. The second rat died on the 14th day with 3 foci of hemorrhage below the hippocampus and 1 above the corpus callosum. The third rat, which died on the 21st day, presented an area of necrosis with a few leuko-

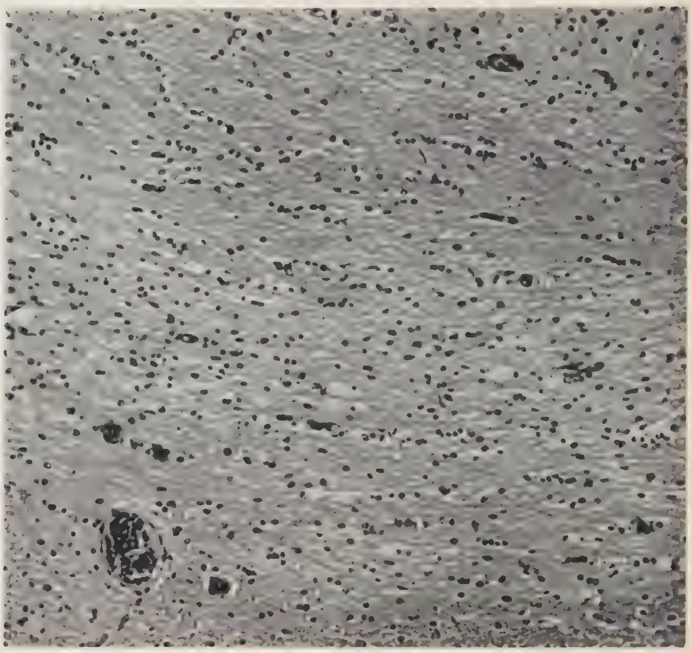

FIG. 2. Cerebral changes in cyanide poisoning. Another rat inj. by subcut. route with daily doses of potassium cyanide. Animal expired on 107th day. Magnification of this brain section is 200×. Normal corpus callosum is seen at upper and lower margins. Center of corpus callosum shows rarefaction, and oligodendroglial cells altered. Number of cells is increased, and cells in general are longer and flatter than those usually seen. Nuclei are darkly stained, details indistinct or invisible.

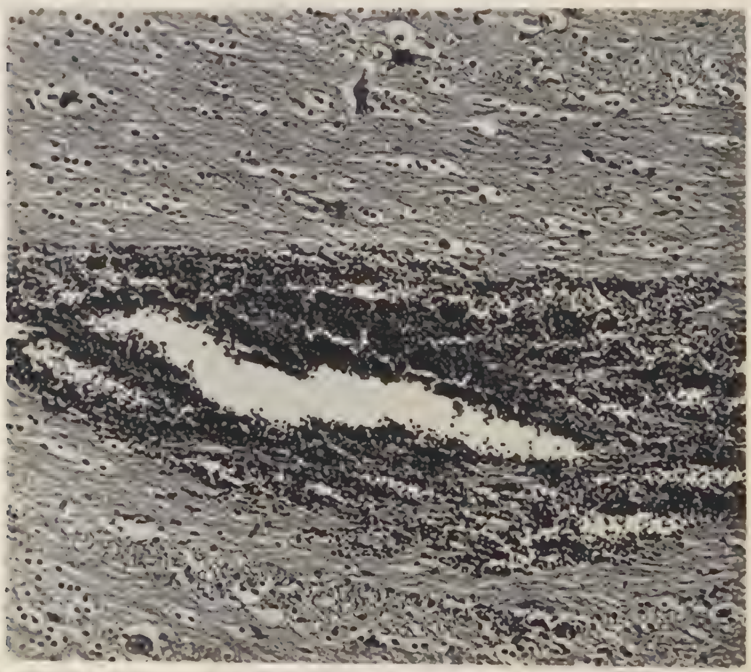

FIG. 3. Cerebral hemorrhage from thiocyanate administration. A rat inj. intraper. with daily doses of potassium thiocyanate. It died on 13th day. Magnification of brain section is 200×. Corpus callosum torn by a region of hemorrhage.

cytes and neuroglial cells, and a small hemorrhagic area in the upper latero-anterior portion of the hippocampus.

B. *Dogs*. 1. *Sodium Cyanide*. Of the 3 dogs injected with daily doses of the cyanide, 1 died on the 10th day; another on the 23rd day; and the 3rd, on the 83rd day. Upon post-mortem examination, the first animal was discovered to have pulmonary edema and congestion with hydrothorax; and the second, pulmonary edema and congestion with slight cloudy swelling of the kidneys and fatty metamorphosis of the liver. The brains of these 2 animals were normal. The third dog survived the longest. Its brain was congested, but there had been no change of behavior during life.

2. *Sodium Cyanide followed by Sodium Nitrite and Sodium Thiosulfate*. Five of the 6 dogs in this series completely recovered during the observation period of 5 days from the effect of multiple lethal doses of cyanide. Each animal received injections of antidotes, sodium nitrite and sodium thiosulfate, 3 or 4 times. No abnormal behavior could be made out. One dog died from the poisoning in spite of treatment. Histological examination of its tissues revealed pulmonary edema and necrosis of some renal tubules. The brain was normal.

Discussion. The data presented indicate that repeated subcutaneous injections of potassium cyanide are injurious to the brain of only 10% of rats. The lesions when present occur in the form of necrosis or hemorrhage. They are located in the corpus callosum or the hippocampus, and may be found at both regions. Demyelinization and focal rarefaction may be present (Fig. 2), the same as reported by Lumsden(8). There is reason to believe, therefore, that cyanide poisoning does not consistently injure the brain.

Three out of 39 rats injected with potassium thiocyanate alone also showed brain lesions. Since thiocyanate is the detoxification product of cyanide when animals are treated with sodium thiosulfate(1), it is not improbable that this substance may be responsible for cerebral lesions. Even without treatment thiocyanate is formed by the natural sulfur donors such as cystine and glutathione. Thus prolonged cyanide poisoning without treatment, or continuous administration of thiocyanate in rats, may result in brain injury although only in a few animals (10-13%). However, this reaction might be due to the high susceptibility of few rats since it occurs rarely and is not dependent on the dosage. In spite of the larger quantity of potassium thiocyanate administered than that formed from potassium cyanide in the body, the incidence of cerebral damage amounts to about 10% in both series of experiments. Our results indicate that sodium nitrite does not cause pathological changes in the central nervous system of the rat.

In dogs the results are much less conclusive or actually negative. Only 1 out of 3 animals receiving daily doses of sodium cyanide showed some congestion of the brain. Of the 6 dogs treated with the nitrite-thiosulfate therapy during severe cyanide poisoning, 1 died but had no cerebral lesions. None of the 9 animals exhibited any abnormal behavior simulating psychotic manifestations. Admittedly, our series of dogs was small; but the negative results tend to minimize the possibility of damage caused by the antidote to the central nervous system. Our results in animals, thus, throw little light on the excitement and violence of Williams' 2 cases(1,2). Since the psychiatric background of the 2 patients was not explored, it may be that their hyperactivity after recovery was merely coincidental with cyanide poisoning.

Summary. 1. Prolonged cyanide poisoning in rats may cause cerebral changes in 10% of the animals. 2. Daily injection of potassium thiocyanate similarly results in brain injury in 10% of rats. 3. In the rat continuous administration of sodium nitrite does not produce lesions in the central nervous system. 4. In dogs cyanide poisoning with or without the nitrite-thiosulfate therapy does not induce abnormal behavior. When they die from the poisoning, their brains usually show a normal appearance.

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Protection of Mice from Neurotoxic Action of Influenza Virus by "Heat Inactivated" Receptor Destroying Enzyme.* (21468)

VINCENT GROUPÉ, ERNEST C. HERRMANN, JR., AND ROBERT M. DOUGHERTY.

From Institute of Microbiology, Rutgers University, State University of New Jersey, New Brunswick.

In attempting to differentiate the microbial product xerosin(1), produced by *Achromobacter xerosis,* from receptor destroying enzyme (RDE) produced by *Vibrio cholerae*(2) comparative studies of these two preparations were made. It will be recalled that both RDE (3) and xerosin(4) are capable of protecting mice from the neurotoxic effect of influenza virus for mice described by the Henles(5), although xerosin failed to alter hemagglutination by influenza virus(6). Surprisingly, it was found that partially purified RDE, heated under conditions that destroyed its capacity to render chicken erythrocytes unagglutinable by influenza virus, was still capable of protecting mice from the neurotoxic action of influenza virus. Because of the wide applications of RDE in the study of the influenza group of viruses the data are presented here.

Methods. The PR-8 strain of influenza A virus and the LEE strain of influenza B virus were used. Partially purified RDE was generously contributed by Dr. John Spitzizen of the Medical Research Laboratories of Sharp and Dohme, Inc. The particular preparation used in these studies contained 200 units/mg as tested in the hemagglutination inhibition test commonly employed for assay of such preparations(7). Physiological saline containing 0.1% $CaCl_2$ was used as diluent in all experiments in which RDE was employed. The preparation of allantoic fluids containing toxic quantities of influenza A virus, the method of intracerebral inoculation, and the technic of inducing convulsions by twirling the mice were identical with those described by the Henles(5). Protection tests in mice were performed in a manner similar to that used by Wagner(3).

Results. A typical experiment illustrating the comparative protective effect of unheated and heated RDE in mice subsequently inoculated with toxic quantities of influenza virus is described below. In this experiment a 1.0% solution of partially purified RDE in saline containing 0.1% $CaCl_2$ was divided into two equal parts. One-half served as the unheated control solution. The other half was placed in the autoclave and heated to 120°C for 30 minutes. The hemagglutination inhibition titer of each solution was determined in the usual fashion(7). The protective activities of the control and heated solutions for mice were compared in the following manner. Serial decimal dilutions of each solution were made and 0.01 ml amounts of each dilution were injected intracerebrally into groups of 10 mice. Twenty-four hours later these mice and 20 control mice were each inoculated intracerebrally into the opposite hemisphere with 0.03 ml of toxic influenza A virus (undiluted allantoic fluid). Daily thereafter for 3 days, each mouse was suspended by its tail and twirled vigorously to induce tonic convulsions. The results are summarized in Table I. It will be seen that although the hemagglutination inhibition (H.I.) titer of

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TABLE I. Protection of Mice against Neurotoxic Action of Influenza A Virus by Heat Inactivated RDE.

µg of RDE inj. intracer. 24 hr before virus*	Partially purified RDE Unheated	Partially purified RDE 120°C for 30 min.
100	0/10†	0/10
10	0/10	0/10
1	0/10	2/9
0.1	6/9	7/10
0.01	5/10	7/10
Saline	17/20	
H.I.‡ titer of 1% sol. of RDE	1/2048	0

* 3.0 LD$_{50}$ of influenza A neurotoxin intracer.
† No. of mice induced to convulsions/Total.
‡ H.I. = Hemagglutination inhibition titer.

partially purified RDE was reduced from 1/2048 to 0, the protective activity in mice was apparently reduced to some extent but was certainly not destroyed.

It is of interest to recall that Wagner(3) postulated the existence of an active impurity in partially purified RDE and that xerosin, another partially purified microbial product, has also been shown to protect mice under identical conditions(4).

Summary and conclusion. 1. Exposure of a solution of partially purified receptor destroying enzyme (RDE) to a temperature of 120°C for 30 minutes completely inactivated its erythrocyte receptor destroying activity but failed to inactivate its capacity to protect mice from the neurotoxic effect of influenza A virus. 2. It is concluded that heat denatured RDE is either still capable of protecting mice or that partially purified RDE contains a heat stable impurity that is capable of protecting mice but that does not affect hemagglutination.

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Comparative Studies of Lipoproteins in Various Species by Paper Electrophoresis.* (21469)

Elaine T. Bossak, Chun-I Wang, and David Adlersberg.
From the Departments of Medicine and Chemistry, Mount Sinai Hospital, New York City.

Paper electrophoresis provides a technic by which serum proteins and lipoproteins may be studied simultaneously(1,2,3). Combined with chemical estimation of individual lipid fractions, detailed analyses of normal and pathologic sera are feasible(4,5). This combined method of study was first applied to establish normal species patterns in man and laboratory animals and extended to investigation of pathologic states.

Procedure. Paper electrophoresis was performed with a horizontal apparatus with free suspension of the paper strips. Six strips of Whatman 3MM filter paper, 2.5 x 45 cm, were run simultaneously. Veronal buffer at pH 8.6 and 0.05 ionic strength was used. After initial equilibration, serum was applied by streaking across a ruled center line on the paper: .005 cc was used for protein determinations and .03 to .06 cc was applied to parallel strips for lipid determinations. A constant voltage of 250 volts was employed for 7 hour runs, with a current of 10-15 milliamperes. After drying at 70° for 30 minutes parallel strips were stained with Amidoschwarz 10B(6) (washed

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in 30% acetone for 30 minutes), and with Oil Red O(7). Densitometry was performed with a Photovolt Model 525 instrument. Optical density was recorded at millimeter intervals. Protein and lipid patterns of serum run in parallel were plotted on one graph for comparison of the resultant curves. These were analyzed by planimetry. Chemical lipid partitions involved determination of cholesterol and esters by the method of Schoenheimer and Sperry(8), phospholipids by the Sperry modification of the Fiske-SubbaRow method(9) and total lipids using Bloor's procedure(10). Neutral fat was calculated by subtracting the figure for total cholesterol plus phospholipids from the value for total lipid. Determinations were performed in humans and monkeys on serum and in rabbits and dogs on heparinized plasma. Blood was obtained after a 16 hour fast and the serum or plasma examined fresh or after brief storage in the frozen state. The human beings studied were of both sexes, ranging in age from 18 to 45 years, and clinically free of disease. Ten runs were performed, nine on pooled sera of three specimens each, to total 28 sera. Three male and two female Rhesus monkeys were included; they were maintained on a diet of fruits, vegetables, whole wheat bread and pellets.† Eight normal male mongrel dogs (weight 11-15 kg) were examined; they were maintained on a diet of meat, vegetables and milk. Seven runs were performed, one on pooled plasma from two animals. Fourteen normal male Chinchilla rabbits (weight about 3 kg) were used; they were maintained on a diet of Purina chow. Twelve runs were performed, one on pooled plasma from 3 animals. Chemical lipid determinations were done on pooled plasma from groups of 3 to 5 rabbits. The pathologic group included 2 male monkeys maintained on a synthetic diet deficient in pyridoxine. Two male dogs which had been rendered myxedematous by thyroidectomy were studied.† Two dogs (#202 and #205) were examined after feeding daily supplements of 8 g of cholesterol U.S.P. (Armour) for 2 and 6 weeks. These 2 animals plus 2 others (#204 and #206) also maintained on cholesterol supplements were studied after daily injections of 200 mg of cortisone for 1 to 4 weeks. Two male rabbits fed daily 1 g of cholesterol chow (without fat-vehicle) for 10 weeks were included(11). In addition, 2 rabbits were examined on the cholesterol diet plus 1.25 mg/kg of cortisone injected daily for 3-4 weeks.

Results. The lipoprotein curves depicted in Fig. 1 with their corresponding paper strips have been analyzed into 3 components: α-lipoprotein corresponding mainly to α-1 globulin on the protein curve and including some α-2, β-lipoprotein corresponding to β globulin on the protein curve, and O—fraction adsorbed at the origin. Average figures for each component and chemical lipid analyses are given for each species in Table I.

Man was characterized by high levels of all serum lipid fractions, particularly of total and esterified cholesterol, with a cholesterol: phospholipid ratio of .83. The serum lipoproteins were distributed with about ⅓ as α lipoprotein and ⅔ as β + O.

The plasma lipids of the dog were characterized by an average level of total and esterified cholesterol somewhat lower than that of man, with phospholipids considerably higher, resulting in a cholesterol:phospholipid ratio of .56. The plasma lipoprotein profile of the dog was almost exactly the reverse of that of man, with α lipoprotein representing 70% of the total.

Analysis of normal monkeys yielded values for total and esterified cholesterol which were considerably lower than those observed in man and dog, with intermediate levels of phospholipids resulting in a cholesterol:phospholipid ratio of .49. Serum lipoprotein distribution was similar to that found in man, with somewhat higher values for α lipoprotein and corresponding slight decrease in β lipoprotein + O.

Plasma from normal rabbits contained considerably smaller amounts of all lipid fractions. Cholesterol:phospholipid ratio was .5. Lipoprotein distribution was similar to that in human serum: the main component was β + O with much smaller amounts of α lipoproteins.

Differences in mobility of albumin among

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† Provided through the courtesy of Dr. Gladys Emerson, Merck Research Institute, Rahway, N. J.

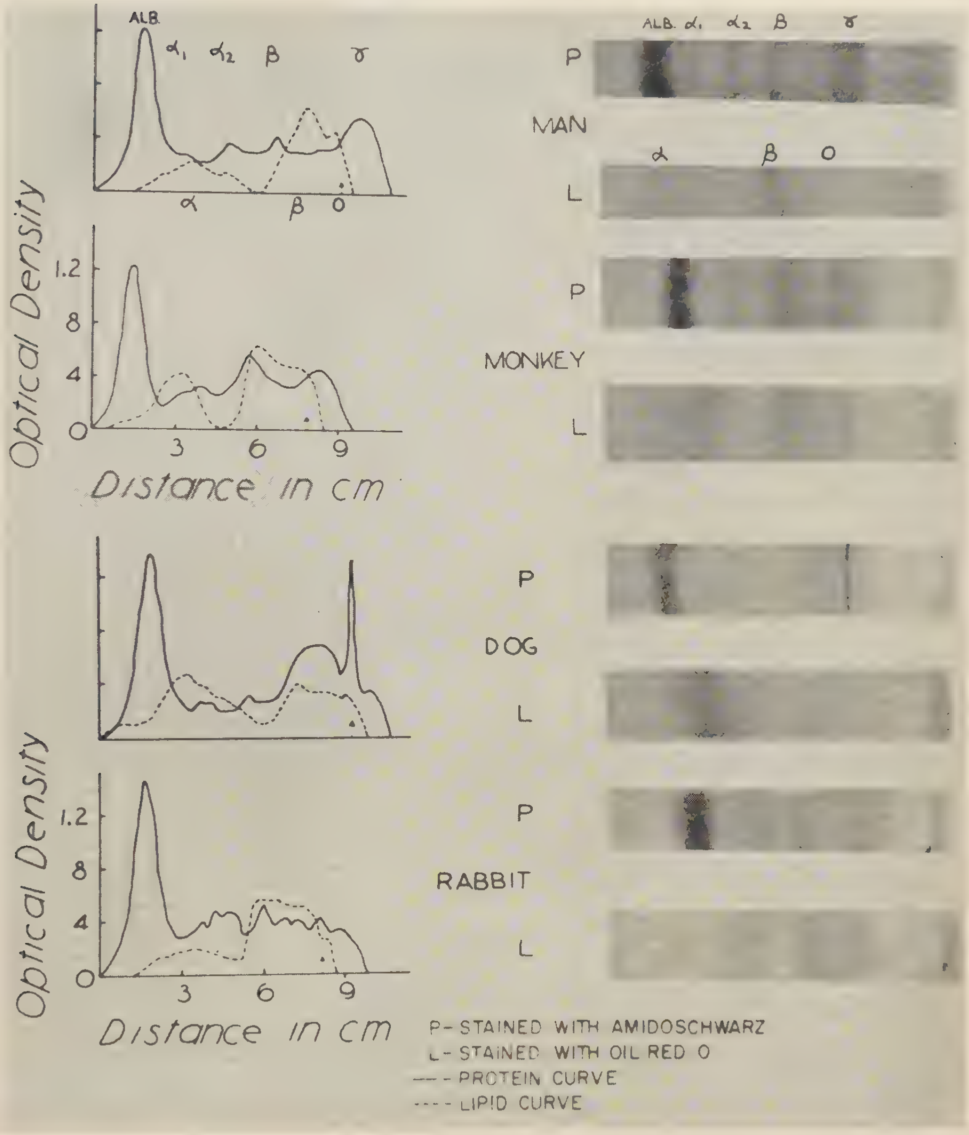

FIG. 1. Protein and lipoprotein electropherograms in normal man, monkey, dog and rabbit. On left are curves obtained by densitometry; on right are stained paper strips. P = paper strip stained for protein; L = paper strip stained for lipid. Arrow in graphs and ruled line on paper strip represent the point of application of the serum. For details, see text.

the various species were noted. Canine albumin had the most rapid mobility, followed by human, rabbit and monkey albumins (Fig. 1). Protein patterns were otherwise similar except for marked predominance of β globulin in dog plasma and separation of rabbit plasma into multiple α and β components.

Examination of sera from pyridoxine-deficient monkeys revealed a moderate lowering of total and esterified cholesterol and total lipid with marked decrease in phospholipids and slight increase in neutral fat. There was no significant alteration of the lipoprotein profile (Table II). These animals lost considerable weight, displayed anorexia, change in body hair and thickening of the intima of the arteries(12).

In dogs rendered myxedematous by thy-

TABLE I. Lipoprotein Profile and Lipid Partition in Various Species.

Species	No.	α	β + O	Cholesterol /esters	Phospholipid	Neutral fat	Total lipid
		% of total stainable lipid		mg/100 ml			
Man	1	45.0	55.0	234/174	255		
	2	28.8	71.2	227/175	267		
	3	41.7	58.3	183/128	258		
	4	37.8	62.2	218/162	293		
	5	29.5	70.5	194/145	237		
	6	24.2	75.8	169/127	228		
	7	30.0	70.0	270/199	299		
	8	39.4	60.6	235/177	233		
	9	29.2	62.8	198/153	231		
	10	39.6	60.4	182/128	230		
Mean		35.3 ± 6.7	64.7 ± 6.7	211/157 ±31 ±25	253 ± 26	165	629*
Monkey	101	45.8	54.2	137/ 99	316	367	820
	102	40.9	59.1	143/102	316	321	780
	103	36.2	63.8	166/146	250	304	720
	104	39.3	60.7	105/ 85	230	365	700
	105	42.0	58.0	143/100	294	443	880
Mean		40.8 ± 3.5	59.2 ± 3.5	139/106 ±22 ±39	281 ± 39	360 ± 54	780 ± 73
Dog	201	74.1	25.9	182/147	310	403	895
	202	67.5	32.5	116/ 95	236	163	515
	203	70.3	29.7	160/124	316	144	620
	204	65.4	34.6	198/144	326	176	700
	205	60.1	39.9	232/173	336	387	955
	206	77.0	23.0	160/117	336	249	745
	207	73.7	26.3	176/134	335	414	925
Mean		69.7 ± 5.8	30.3 ± 5.8	175/133 ±36 ±25	314 ± 36	276 ± 56	765 ± 53
Rabbit	301	41.7	58.3				
	302	46.2	53.8	33/ 24	94	163	290
	303	40.9	59.1				
	304	35.2	64.8				
	305	34.6	65.4	89/ 65	125	311	525
	306	29.6	70.4				
	307	26.7	73.3				
	308	23.0	77.0				
	309	32.9	67.1	58/ 49	115	252	425
	310	30.0	70.0				
	311	23.5	76.5				
	312	21.7	78.3	62/ 47	141	317	520
Mean		32.2 ± 7.9	67.8 ± 7.9	60/ 46 ±21 ±15	119 ± 18	261 ± 65	440±100

* Mean value of 11 normal healthy blood donors.

roidectomy there was considerable elevation of all plasma lipid fractions (Table II). In dog 208 a large α-2 lipoprotein component appeared (28.8% of total lipoprotein), while there was no alteration in the β + O fraction. In dog 209, there was a significant elevation of the β + O fraction with concomitant fall in the level of α lipoprotein.

Cholesterol-fed dogs revealed moderate elevations of plasma total and esterified cholesterol and phospholipids, with less elevation of total lipid and no significant change in neutral fat. There was a slight elevation of β lipoprotein + O and corresponding slight depression in α lipoprotein.

Administration of cortisone to cholesterol-fed dogs resulted in enhancement of the plasma lipid elevation to the highest levels observed in the animals studied (Table II). Levels of serum total and esterified cholesterol averaged 2½ times normal, while levels of all other lipid components were doubled. The plasma of these dogs became lactescent. Marked alteration of the plasma lipoprotein

TABLE II. Lipoprotein Profile and Lipid Partition in Experimental States.

Species	No.	Treatment	α	$\beta+0$	Cholesterol /esters	Phospho-lipid	Neutral fat	Total lipid
			% of total stainable lipid		mg/100 ml			
Monkey	101–5	None*	40.8	59.2	139/ 106	281	360	780
	106	B_6 deficient diet	38.0	62.0	100/ 64	211	389	700
	107	*Idem*	39.3	60.7	70/ 46	124	406	600
Dog	201–7	None†	69.8	30.2	175/ 133	314	276	765
	208	Thyroidectomy	68.5	31.5	270/ 209	391	569	1230
	209	”	52.3	47.7	345/ 260	500	465	1310
	202 A	Cholesterol	64.3	35.7	277/ 206	369	244	890
	205 A	”	60.7	39.3	337/ 246	505	268	1110
	202 B	Chol. + cortisone	37.6	62.4	612/ 430	614	434	1660
	204 B	*Idem*	55.7	44.3	482/ 342	552	311	1345
	205 B	”	49.2	50.8	733/ 466	805	932	2470
	206 B	”	43.8	56.2	277/ 198	460	473	1210
Rabbit	301–12	None‡	32.2	67.8	60/ 46	119	261	440
	313	Cholesterol	2.4	97.6	1208/ 964	384	808	2400
	314	”	.9	99.1	1552/1156	588	2000	4140
	315	Chol. + cortisone	2.9	97.1	2576/1752	972	3472	7020
	316	*Idem*	0	100	1412/1098	512	1136	3060

* 5 animals. † 7 animals. ‡ 12 animals.

pattern was observed with the β lipoprotein + O fraction accounting for more than half of the total sudanophilic lipid (Fig. 2). Alterations of plasma proteins in these animals consisting of hyperglobulinemia with marked elevation of α-2 globulin will be described in detail elsewhere(13).

The effect of cholesterol-feeding on plasma lipids of the rabbit is illustrated in Table II. Cholesterol and esters rose to a value 20 times normal while phospholipids, neutral fat and total lipids increased to 5 times normal. The plasma had the appearance of heavy cream. This was accompanied by extreme changes in the lipoprotein profile. Alpha lipoprotein was decreased to minimal levels being replaced by a marked elevation of the O fraction. Marked atherosclerosis and lipidosis occurred in these animals(14).

The combination of cortisone and cholesterol administration in the rabbit resulted in marked enhancement of the elevation of all lipids, especially of the cholesterol fractions and of total lipid, to the highest values observed in this series (Table II). The lipoprotein profile was completely distorted and again characterized by almost total disappearance of α lipoprotein and extreme elevation of the β + O fraction. It was impossible in one case to separate the O fraction from β lipoprotein (Fig. 3).

Discussion. The combination of paper electrophoresis and chemical lipid determinations permits detailed investigations of normal and abnormal lipid metabolic states. Values for distribution of lipoproteins obtained with this method are similar to those obtained by more elaborate methods such as Cohn fractionation(15) or ultracentrifuge technics(16). These require larger amounts of serum, expensive apparatus and considerable time and prolonged technical training. With paper electrophoresis comparative quantitative studies of lipoproteins are readily available for correlation with chemical analyses of lipids.

The species examined showed distinctive specific patterns of both lipoprotein profile and lipid partition. Man and dog, for example, stand at opposite poles with regard to serum lipoprotein distribution while the low chemical lipid values of the rabbit contrast sharply with those of man.

Man, highly predisposed to spontaneous atherosclerosis, exhibited the highest cholesterol level, highest cholesterol:phospholipid ratio and predominance of β lipoprotein. The rabbit, in which atherosclerosis is most easily induced, exhibited under these experimental conditions extremely high cholesterol values, a very high cholesterol:phospholipid ratio and marked elevation of β lipoprotein. In the

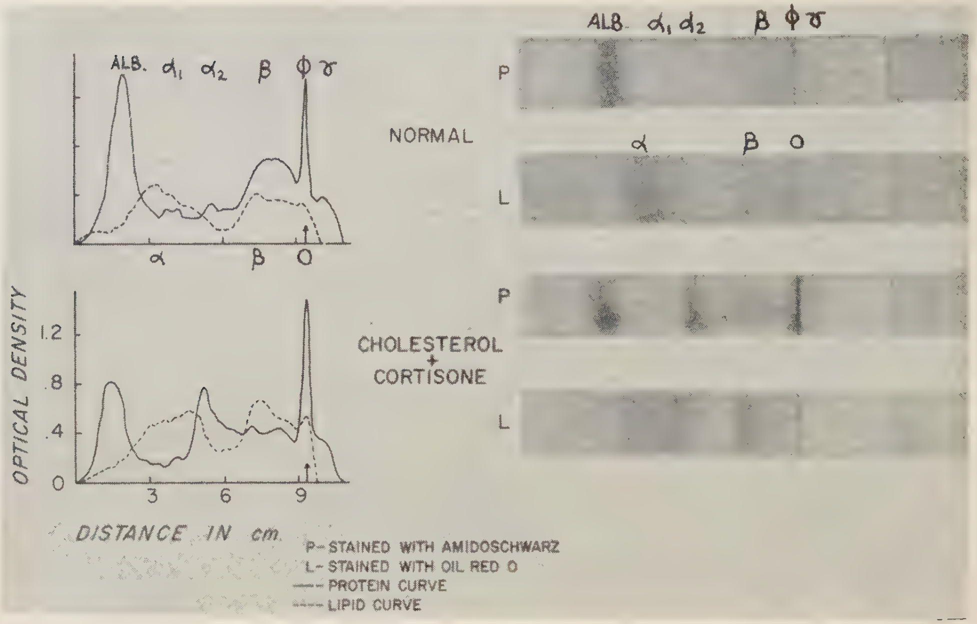

FIG. 2. Protein and lipoprotein electropherogram of dogs on dietary cholesterol supplement plus cortisone injections contrasted to normal dog. For details see legend to Fig. 1 and text.

cholesterol-fed dog receiving cortisone there was a resemblance to the lipid-lipoprotein spectrum observed in man. The failure of these animals to develop atherosclerosis may be related to the comparatively short duration of the experiments, as yet unexplored species differences and perhaps to the effect of cortisone to diminish tissue permeability(17).

Thyroidectomy, cholesterol-feeding and cortisone treatment in the dog were all reflected

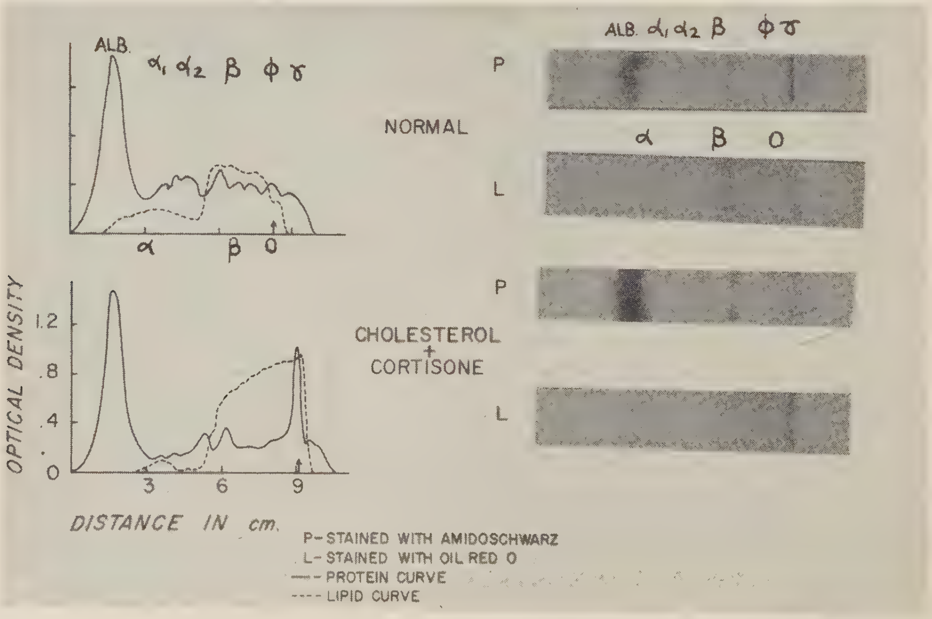

FIG. 3. Protein and lipoprotein electropherogram of rabbits on dietary cholesterol supplement plus cortisone injections contrasted to normal rabbit. For details see legend to Fig. 1 and text.

by increase in β lipoprotein. As yet, no pathologic elevation of α lipoprotein, the predominant fraction in this species, has been observed.

Summary. 1. Paper electrophoresis provides a simple reproducible method for the study of serum (plasma) lipoproteins. Characteristic species lipoprotein patterns were demonstrated in normal man, monkey, dog and rabbit. 2. This method combined with chemical determinations of serum (plasma) lipids permits detailed investigations of normal and abnormal lipid metabolic states. 3. Elevation of serum (plasma) lipid fractions induced in these species by various experimental procedures were accompanied by a lowering of α lipoprotein and varying degrees of elevation of the β + O fraction.

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Quantitative Determination of Ethanolamine Phosphate and Free Amino Acids of Normal and Neoplastic Tissue. (21470)

L. C. SMITH AND FRANCES M. ROSSI. (Introduced by Smith Freeman.)
From Research Division, Veterans Administration Hospital, Hines, Ill., and Department of Biochemistry, Northwestern University Medical School, Chicago, Ill.

Inasmuch as the free amino acids of tissue may contribute to the synthesis of protein, their concentration in normal and tumor tissue is of considerable importance. Nakahara, *et al.*(1), could not show any significant differences between non-protein nitrogen or free amino nitrogen in certain tumors and their homologous normal tissues. Other workers (2,3) have demonstrated that tissue of certain tumors has a higher concentration of free arginine than normal control tissue. Roberts and Frankel(4) studied by 2-dimensional chromatography the patterns of free amino acids in mouse tumors and normal homologous tissue. Their qualitative studies showed that each normal tissue has a distribution of free amino acids which is distinctive and characteristic of that tissue, and that tumor tissue also, regardless of its source, showed a recognizable and distinctive free amino acid pattern. Kit and Awapara(5) were the first to report quantitative determinations of amino acids in a number of neoplasms and normal tissue. Using the paper chromatographic method of Awapara(6), they were able to elute the ninhydrin spots and determine the amounts of several amino acids with a fair degree of accuracy. Their results showed that the amino acid pattern of neoplasms was drastically altered as compared to that of normal lymphatic tissue. Lymphosarcomas exhibited markedly elevated contents of alanine and glycine. Proline was also elevated in these tissues compared to the normal tissues.

It appears that a continuation of these studies of the free amino acid concentration of tissues, and the relation of these to metabolic activity and growth rates should be of considerable importance in elucidating the role of such intermediates in protein synthesis. Since continued and unrestrained growth is characteristic of malignant tissue, such studies are of particular interest for the understanding of the chemistry and metabolism of cancer. The studies reported in this communication describe the free amino acid levels as found in 2 rat tumors, 3 mouse tumors, and 3 normal tissues.

Material and methods. The transplantable Bagg reticulum cell sarcoma and Walker carcinoma tumors of Sprague-Dawley rats were used. These along with normal rat thymi and testes, and rabbit thymi were prepared as follows: fat, mucoid and necrotic materials were trimmed away. The tissues were then finely minced with scissors and passed through a #80 Monel Screen. The result was a suspension of free cells which were then placed in Ringer-Phosphate solution. Mouse Ehrlich Carcinoma cells, and Sarcoma cells #180 and #37 were grown as free suspensions in the ascitic fluid of mice. These were aspirated by medicine dropper, the cells removed by centrifugation and placed in Ringer-Phosphate solution. All cells were then washed twice with Ringer-Phosphate solution to remove any adhering amino acids. The viable and dead cells were then counted by the method of Schrek (7). Aliquots were then removed for dry weight determinations. Cells were treated with 10% T.A.A. to remove the proteins and to release the free amino acids from the ruptured cells. After removal of the T.A.A. by ether extraction, the resulting solution was concentrated *in vacuo* and made up to a 5 ml volume. *Quantitative amino acid determinations* were performed by the Dowex-50-citrate buffer method of Stein and Moore(8). The effluent was collected in 1 ml fractions by use of a Technicon fraction collector and color developed with 2 ml of ninhydrin solution. Determinations made on synthetic mixtures indicated recoveries of 97%.

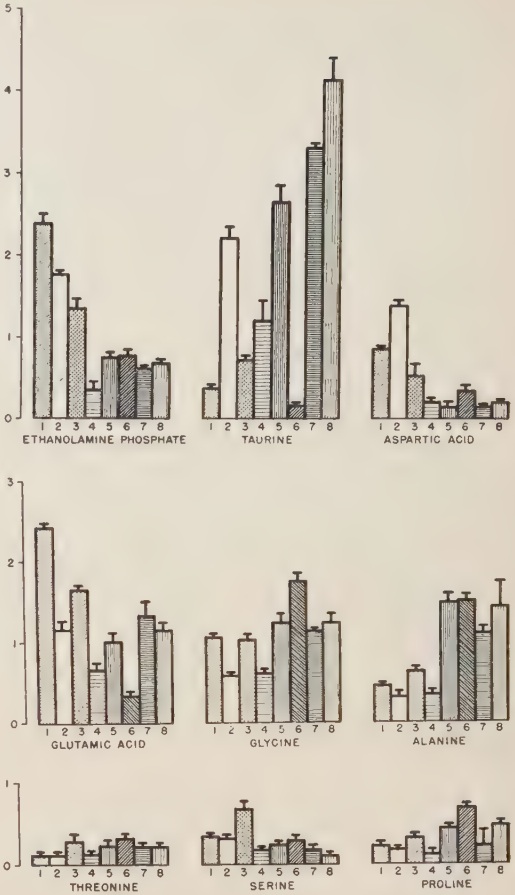

FIG. 1-3. Amino acid concentrations of normal and neoplastic tissues. Ordinate: mM/100 g dry tissue. Height of each bar represents the mean concentration. The probable error of the mean result is shown above each bar. Numbers refer to: 1. Rabbit lymphocytes; 2. rat lymphocytes; 3. rat testicular cells; 4. Walker ca. cells; 5. S-37 sarcoma cells; 6. reticulum sarcoma cells; 7. S-180 sarcoma cells; 8. Ehrlich ca. cells.

Results. The distribution of free amino acids in the various normal and neoplastic cells are shown in Fig. 1-3. The values are expressed as mM acid/100 g of dry cells. The results show that the amino acid pattern of a given tissue is fairly constant; however it may vary considerably from another tissue.

Although ethanolamine phosphate has been reported to occur in both normal animal tissues and tumors(9,10,11,12), it has not been previously separated and determined by column chromatography using Dowex-50 resin. Using a 0.9 x 100 cm column, and a sodium citrate buffer of pH 3.42, it was found that this compound was resolved in a position intermediate between cysteic acid and taurine. It gave a very sharp peak, usually being confined to tubes 28-32. Thus, its very acidic properties are apparent since the hold up volume of this size column is about 22 ml. The substance was identified by its stability to acid hydrolysis, and its phosphorous content as determined by the method of Gomori(13). Final proof was furnished by the synthesis of aminoethylphosphoric ester according to the method of Outhouse(14). This was run on the column separately and with a tissue extract. The unknown peak of the extract was found to overlap exactly the peak of the synthetic compound. Color yields were made on a molar basis relative to leucine similar to that done by Moore and Stein(8). The color yield was found to be 1.00.

In the case of normal tissue, a species difference is noted for several amino acids. This had previously been pointed out by Kit *et al.* (15) in the case of taurine. The rabbit lymphocytes have considerably more ethanolamine phosphate, glutamic acid and glycine than rat lymphocytes, while the latter are much richer in taurine and aspartic acid. The total amino acids for the 2 similar cells from different species are very close, being 8.31 ± .06 for the rabbit and 8.2 ± .15 for the rat.

The rat testicular cells were chosen in order to obtain actively dividing cells. The results show that these cells are quite rich in ethanolamine phosphate, glutamic acid and glycine. The taurine and aspartic acid contents are much lower than that found in the rat lymphocytes; however, the alanine and glycine were approximately twice as large.

Neoplastic cells. The patterns shown by the Ehrlich carcinoma cells and the S-180 and S-32 sarcoma cells were quite similar. The former had a larger total, but most of this rise could be attributed to the consistently higher taurine content. All these tumors showed high and similar concentrations of glutamic acid, glycine, taurine and alanine. Although the ascites tumors consisted of 2 entirely different types of neoplastic cells, *viz*: sarcoma *vs*. carcinoma, as pointed out, the amino acid pattern was quite similar. However, in the case of the solid tumors, considerable differences were noted between the Bagg reticulum sarcoma and Walker carcinoma cells. The total amount of amino acids in the reticulum cells was approximately 1.6 times as great as the Walker cells. Alanine and glycine were the dominant acids present in the sarcoma cells, followed by somewhat smaller amounts of proline and ethanolamine phosphate. Taurine made up the smallest amount of the amino acids determined. This is in contrast to the findings of Kit and Awapara(5) who used 4 different lymphosarcomas and consistently found taurine to be one of the dominant amino acids of these tissues.

Discussion. The results noted in this work are similar to those pointed out by Kit and Awapara(5), for lymphosarcomas, namely, that there are high concentrations of alanine in the various tumors. The one exception noted was that of Walker Carcinoma cells. On the other hand, glycine appears to be just as abundant, and in most cases even greater than alanine. Taurine, with the one exception of the lymphosarcoma, was by far the most abundant of the amino acids determined. Although its physiological significance in various tissues is not known, it is interesting to speculate on its origin. Do the large amounts arise in other tissues, for example, the liver? Then by a method of active concentration, as shown by Christensen(16) *et al.* for the ascites carcinoma cell, is the metabolite more or less irreversibly "trapped" by certain cells? Or is it being actively produced from the metabolism of the sulfur containing amino acids within the cell where found? These questions must remain unanswered for the time being.

Similar questions may be asked of the compound, aminoethylphosphoric ester. Its role in tissue metabolism is still obscure. It is interesting to note that the acid does not disappear during autolysis, which means that normal tissue phosphatases do not split it readily(17). Chargaff and Kesten(18) concluded that the ester is not used for phosphatide formation, but rather results from the breakdown of phosphatides. On the other hand, Ansell and Dawson(19) have shown that the major portion of the acid did not originate by the breakdown of phosphatidyl ethanolamine in rat brain. On the contrary, they were able to demonstrate the synthesis of ethanolamine phosphoric acid by isolated rat-brain tissue. Possibly ethanolamine phosphate is formed by the mechanism proposed by Luck and Wilcox(20), *i.e.*, serine → phosphoric ester → ethanolamine phosphoric ester. In any case, the origin, role, and fate of this rather abundant tissue metabolite remain obscure at this time.

The relatively low content, with a few exceptions, of aspartic acid, threonine, serine and proline might indicate that these amino acids are either not as actively concentrated as the others, or that they are more rapidly utilized.

Summary. 1. Quantitative measurements have been made by Dowex-50 chromatographic columns of the free amino acid content of the free cells of: rat thymi and testes, rabbit thymi, Ehrlich ascites carcinoma cells, mouse sarcoma cells, S-37 and S-180, Bagg reticulum cell sarcoma and Walker carcinoma. 2. The results indicate that taurine, ethanolamine phosphate, glycine and glutamic acid are the most abundant of the amino acids determined. 3. Proline, aspartic acid, serine and threonine occur in rather small quantity. 4. Alanine seemed to vary in quantity in the various cells. 5. The results were discussed in terms of the metabolic reactions which lead to the origin and fate of certain of these metabolites.

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Assay of Plasma Prothrombin with a Synthetic Substrate.* (21471)

HELEN I. GLUECK, SOL SHERRY,† AND WALTER TROLL.‡
(With the technical assistance of Shirley Frank.)

From the May Institute for Medical Research, Jewish Hospital Assn., and Department of Medicine, University of Cincinnati College of Medicine.

In a recent communication, evidence has been presented for the hydrolysis of arginine esters by thrombin(1). P-Toluenesulfonyl-l-arginine methyl ester (TAMe) has been found particularly suitable for the measurement of thrombin(1,2). Based on this observation, a

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† Present address: Division of Medical Services, Jewish Hospital of Saint Louis, St. Louis, Mo.

‡ Present address: Cancer Research Institute, New England Deaconess Hospital, Boston, Mass.

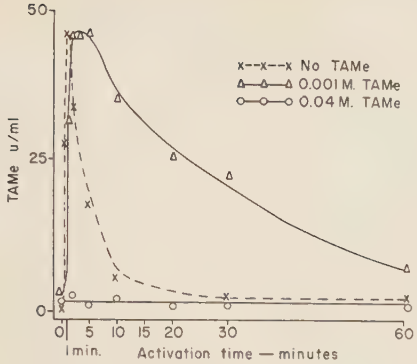

FIG. 1. Activation of plasma prothrombin by thromboplastin and calcium in presence of no TAMe, 0.001 M TAMe, and 0.04 M TAMe.

method for the quantitative assay of prothrombin in plasma has been developed, and measurements have been made of the plasma prothrombin in normal and abnormal states.§

Method. When prothrombin is converted to thrombin by calcium and thromboplastin, the presence or absence of TAMe in the activation mixture exerts a marked effect on the results. If no TAMe is present, the thrombin evolving from prothrombin is rapidly inactivated by antithrombin. Quantitative measurement of prothrombin under these circumstances depends on the time of sampling (Fig. 1). If, however, dilute TAMe (0.001 M final concentration) is present in the activation mixture, the conversion of prothrombin to thrombin is complete in 2 to 3 minutes provided that the plasma contains a moderate amount of Ac-globulin. The thrombin evolved under these circumstances, however, is protected from destruction by antithrombin for an additional 5-10 minutes. If the concentration of TAMe in the activation mixture is raised to 0.04 M (final concentration) no thrombin can be evolved from prothrombin. A previous communication from this laboratory has discussed the possible sites for the inhibitory effect of TAMe on the conversion of prothrombin to thrombin(2). Although the answer to this problem is incomplete, the observation itself has been utilized in the present method of prothrombin assay. Thus, the presence of 0.001 M TAMe in the activation mixture of the test sample stabilizes and protects the thrombin evolving from prothrombin. The complete inhibition of prothrombin activation in the presence of 0.04 M TAMe is utilized to provide an adequate blank containing all the reagents. The method of thrombin assay is similar to that previously described(1).

Technic of assay. The technic of assay is as follows: 9 volumes of blood are collected with one volume of 0.2 M sodium citrate. Each 1.8 ml of plasma is defibrinated by the addition of 0.2 ml of 100 units/ml of thrombin solution.‖ After removal of the clot with an applicator stick the plasma stands at room temperature for one-half hour. *Blank.* To 0.2 ml of 0.4 M TAMe (M.W. 373)¶ in Tris buffer (0.1 M tris(hydroxymethyl) aminomethane M.W. 121** adjusted to pH 9 with 0.1 N HCl)†† in a 15-ml centrifuge tube, add 0.9 ml of buffer, 0.3 ml of thromboplastin‡‡ in buffer, 0.3 ml of 0.02 M $CaCl_2$ in buffer, and 0.3 ml of defibrinated plasma. Incubate 30 minutes at 37°C. Add 2.0 ml of 37% formaldehyde which has previously been adjusted to a faint pink with sodium hydroxide in the presence of phenolphthalein. *Test.* To 0.2 ml of 0.009 M TAMe in buffer add 0.7 ml of buffer, 0.3 ml of thromboplastin in buffer, 0.3 ml of 0.02 M $CaCl_2$ in buffer, and 0.3 ml of defibrinated plasma. Incubate 30 minutes at 37°C. Add 2.0 ml of 37% formaldehyde which has previously been adjusted to a faint pink with sodium hydroxide in the presence of phenolphthalein. *Test.* To 0.2 ml of 0.009 M TAMe in buffer add 0.7 ml of buffer, 0.3 ml of thromboplastin in buffer, 0.3 ml of 0.02 M

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§ Although plasmin, the proteolytic enzyme of plasma, also hydrolyzes TAMe(1), observations on plasminogen, the naturally occurring precursor, reveal that it is not significantly activated by calcium and thromboplastin during the experimental period, and does not interfere with the estimation of plasma prothrombin.

‖ Prepared from refrigerated stock, 5,000 u/ml thrombin solution (Parke, Davis) in 50% glycerine-saline mixture.

¶ Obtained from H. M. Chemical Co., Santa Monica, Calif.

** Eastman Kodak.

†† All subsequent references to buffer are to 0.1M Tris buffer, pH 9.0.

‡‡ Difco, prepared according to manufacturing direction, but in Tris buffer.

$CaCl_2$ in buffer, and 0.3 ml of defibrinated plasma.[§§] After 5 minutes activation at room temperature,[‖‖] add 0.2 ml of 0.4 M TAMe in buffer and incubate for 30 minutes at 37°C. At the end of incubation, add 2 ml of formaldehyde. Both tubes are centrifuged for 5 minutes at 2,000 rpm. Three ml aliquots of the clear supernatants are then transferred to 25-ml Erlenmeyer flasks. Titration is carried out with 0.05 M sodium hydroxide using a syringe type micro-burette. Two-tenths ml of 0.01% phenol red serves as the indicator. *Calculation.* The TAMe unit has been arbitrarily defined as that amount of thrombin which will release 1 μm of acid from TAMe in 10 minutes under the conditions of the test. The TAMe unit is approximately equivalent to the activity of one N.I.H. clotting unit of thrombin(1). The following equation is used to calculate the number of TAMe units in one ml of plasma: (titration of test in ml—titration of blank in ml) × 91.4.[¶¶] Results of the TAMe assay of prothrombin in normal plasma as a function of the amount of plasma in the test mixture, are seen in Fig. 2. Identical values are obtained when the plasma is diluted with saline or adsorbed prothrombin free plasma. A direct linear relationship is apparent between 0.025 ml to 0.3 ml of normal plasma,[***] in contrast to the usual curvilinear assay of thrombin or prothrombin when measured by available clotting methods.

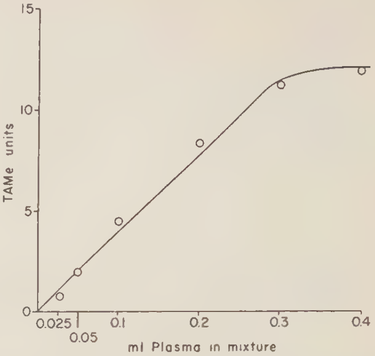

FIG. 2. Quantity of plasma prothrombin, measured by TAMe assay, as a function of amount of plasma in test mixture.

Results. The mean prothrombin content of the plasma of 25 normal subjects studied by the TAMe assay was 53.3 TAMe units with a S.D. of ±4.5. The range in values was 44-62 units/ml of plasma.

The prothrombin concentration of the plasmas of an additional 9 patients receiving dicumarol for thromboembolic disease was determined by both the TAMe and the one-stage assay of Quick(3). In the majority of patients when the plasmas were assayed by the one-stage method, the prothrombin concentration usually fell to 20-30% of the initial concentration by the 4th or 5th day. When

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[§§] Values are consistently lower if whole rather than defibrinated plasma is used. This can not be ascribed to the additional TAMe splitting activity of the thrombin used for defibrination. Plasma which has stood for one-half hour after defibrination does not show an increased spontaneous hydrolysis of TAMe. A more probable explanation is the competitive inhibiting effect of fibrinogen on the TAMe splitting activity of thrombin(1).

[‖‖] Preliminary studies with aged plasma indicated that under conditions of the method, where activation is allowed to proceed 5 minutes, a considerable deficiency in Ac-globulin must exist (prothrombin assays of 35% or less by the 1-stage method of Quick(3)) before a significant reduction in total thrombin yield occurs. The effect of SPCA (convertin) deficiency on total thrombin yield during 5 minutes activation period appears to be less striking. Plasmas, obtained from dicumarolized patients, which respond in the 1-stage method of Quick to the addition of SPCA were used as a source of SPCA deficient plasma. Addition of SPCA to these specimens did not influence the TAMe assay until the 1-stage assay had fallen to 20% or less. At these low levels of "prothrombin" addition of optimum concentration of SPCA increased thrombin yield slightly (20% or less).

[¶¶] This factor is derived by multiplying 10/9 (anticoagulant) x 10/9 (defibrination) x 10/3 (aliquot of plasma used) x 4/3 (aliquot of reaction mixture titrated) x 1/3 (reduction to 10 minutes period) x 50 (conversion of standard alkali to micromoles). For routine assays, consideration of the hematocrit in determination of the anticoagulant dilution has not been deemed necessary(4). For more precise calculations especially where the hematocrit is abnormally high, the hematocrit should be taken into account in calculating dilution factor.

[***] Values obtained with 0.025 ml of normal plasma or less may show a slight "lag" in the curve, probably due to insensitivity of a method in this low range.

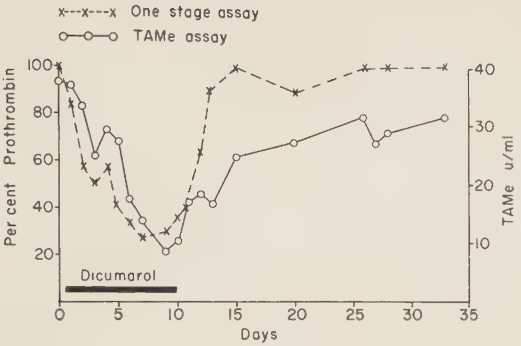

FIG. 3. A comparison of TAMe, and 1-stage assay of plasma prothrombin in a patient receiving dicumarol. Ordinate (left) % prothrombin 1-stage method, ordinate (right) TAMe u/ml plasma. Abscissa represents days of observation.

these same plasmas were assayed by the TAMe method, the disappearance of prothrombin was somewhat less rapid, often requiring 6 to 8 days to reach 10 to 15 TAMe units/ml. Once the prothrombin as measured by the TAMe assay had fallen to 20-30% of the initial concentration there was better agreement between the 2 methods.

On discontinuing dicumarol the disparity between the 2 methods was even more apparent. The plasmas of most patients when assayed by the one-stage method showed a return to pre-treatment level by the 7th day. The same patients followed by the TAMe assay showed a more prolonged depression of their plasma prothrombin. In one patient with thrombophlebitis whose prothrombin (one-stage assay) had returned to normal 8 days after discontinuing dicumarol, the TAMe assay did not reach control levels until 17 days had elapsed. In a second patient (hypertensive cardiovascular disease, congestive failure), 35 days after discontinuance of the drug the TAMe assay of the plasma had not yet reached pre-treatment levels (Fig. 3).

Assay of the plasmas of 6 dogs yielded a mean value of 38 TAMe units/ml of plasma. In 4 rabbits the mean value was 39 TAMe units/ml.

Discussion. The TAMe assay of prothrombin resembles the usual 2-stage assay methods in one important respect. The activation of prothrombin, yielding an optimum amount of thrombin, is carried out separately from the measurement of thrombin(4). It is not unexpected, therefore, that the results obtained with the TAMe assay appear to agree more closely with those reported by the 2-stage methods than those obtained by one-stage assays.[†††]

Shinowara(5) employing a 2-stage adsorption isolation method for prothrombin, found 82.4 ± 17.3 (S.D.) units of prothrombin/ml of human plasma. This value is in good agreement with the TAMe assay of human plasma prothrombin. The TAMe unit can not be compared directly with the clotting unit of the 2-stage method of Ware and Seegers(4) since the TAMe unit, based on the N.I.H. unit in the absence of acacia, equals 2.6 such units when acacia is present (5). Nevertheless, whatever the arbitrary definition of the thrombin unit, it would appear that the TAMe as well as the 2-stage methods both measure the "true" prothrombin of plasma. Murphy and Seegers(6), utilizing the 2-stage method, found that dog and rabbit plasma contain less prothrombin than does the human, although the prothrombin time of dog and rabbit plasma is more rapid than that of the human. The difference between the one-stage and 2-stage assay of plasma prothrombin in animals has been ascribed to a high concentration of accelerator factors in these species(6). The TAMe assay of dog and rabbit plasma prothrombin are in accord with this observation.

The disparity between the one-stage prothrombin assay and the usual 2-stage procedures has been observed previously in dicumarolized plasmas(7-9). These differences have been ascribed to S.P.C.A. (convertin) (10,9) which is presumed to be more sensitive to the effect of dicumarol than prothrombin itself(11-13). The results obtained by the TAMe assay of the plasma of dicumarolized patients would appear to parallel those observed when either the 2-stage procedure of Ware and Seegers(4) or the 2-stage adsorption method of Shinowara(5) are employed.

There appear to be certain advantages to the TAMe assay when compared with clotting methods, for the measurement of plasma prothrombin. The linear relationship between TAMe hydrolysis and prothrombin concentration allows for accurate quantitative assay of prothrombin in a range where clotting technics are difficult; the variability of the reactivity of fibrinogen is eliminated; the activation of prothrombin is complete before the thrombin is assayed, eliminating the necessity of multiple sampling; and high initial dilution of the plasma is unnecessary since the presence of dilute TAMe in the activation mixture inhibits antithrombin.

[†††] Studies comparing the results obtained simultaneously by all three methods, on the plasma of patients receiving dicumarol, are now under way.

Since the TAMe assay only measures plasma prothrombin, it should be emphasized that this method, when used alone, may have intrinsic faults as a clinical test for the management of patients under treatment with dicumarol. Under such conditions the deficit of proconvertin as well as prothrombin, as revealed by one-stage assays, is probably a safer index of therapy.

Summary. A method of measuring plasma prothrombin employing a synthetic substrate (TAMe) has been described. The method has been utilized in studying the plasma prothrombin of normal and dicumarolized patients. Assays of dog and rabbit plasma prothrombin are reported. The results are compared with those obtained by a one-stage procedure.

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Increased Aldosterone Output During Sodium Deprivation in Normal Men.*† (21472)

J. A. LUETSCHER, JR., AND B. J. AXELRAD. (Introduced by A. L. Bloomfield.)
From the Department of Medicine, Stanford University School of Medicine, San Francisco, Calif.

There is a paucity of information on the mechanism by which a normal man's excretion of sodium and chloride is decreased when dietary intake of sodium chloride is reduced. McCance(1) noted impairment of renal function during severe sodium depletion. Smith (2) concluded that either decreased glomerular filtration or increased tubular reabsorption of sodium could reduce sodium excretion in man. Adrenal cortical hormones can increase the reabsorption of sodium by the renal tubules (3), but previous studies have not demonstrated a significant change in the adrenal cortical hormones or their metabolites in the urine of patients on low-sodium diets(4).

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† The assistance of Anne Dowdy, Julia Harvey, Way Lew, and Lee J. Poo is gratefully acknowledged. Determinations of 17-ketosteroids and 17-hydroxycorticoids were done by William Lew, Francis Greenspan, Donald Island and Peter Forsham.

The sodium-retaining corticosteroid, aldosterone(5), has been found in human urine by bioassay(6), separated by chromatography (7), and identified by physical and chemical methods(8). The output of aldosterone appears to be inversely related to sodium excretion both in normal men and in patients with disease(9,10). In the present study, renal function and output of aldosterone and other adrenal cortical hormones have been measured during sodium deprivation in 2 normal men.

Methods. During control and recovery periods, the subjects ate their usual diets. For the 6 days of sodium restriction, the diet was calculated to contain 11 mEq. of sodium per day. Average daily caloric and fluid intakes were unchanged. Small amounts of salt substitute (Neocurtasal®) were used during the experimental period. Urine was collected and measured as 24-hour totals. Aliquots were taken for measurement of sodium and potassium by flame photometer; creatinine chromogens(11); 17-ketosteroids(12); and 17-hydroxy-corticosteroids and metabolites by a modification of Reddy's method(13). In each case, 4 specimens were selected for bioassay, as follows: a pool from 2 control days; day 2 or 3 of sodium deprivation, when urine sodium was falling rapidly; day 5 or 6, when urine sodium was at a minimum; and 2 to 4 days after resuming normal diet. These specimens were acidified to pH 1.0 and extracted within 40 minutes with chloroform (extract A). Each specimen was extracted again after standing for 24 hours at pH 1.0 (extract B). In one case, 2 specimens (a control pool and low sodium day 4) were incubated with β-glucuronidase (mammalian liver, Warner-Chilcott), and extracts G were made. Each extract was washed with cold 0.1 N solution of sodium hydroxide and with cold water, dried over anhydrous sodium sulfate, and freed of chloroform at 40° *in vacuo*. The residue was taken up in ethanol 95% and fractionated by paper chromatography(7). The sodium-retaining activity of the aldosterone fraction was measured by bioassay(14). Results are expressed as the dose of desoxycorticosterone acetate (DCA) equivalent in effect to the dose of urine fraction assayed. Since the activity of crystalline aldosterone is 30 times that of DCA in this bioassay(15), and since the proportion of each day's urine bioassayed is known, the quantity of aldosterone extracted from one day's urine may be calculated. From 1.8 to 3.5 μg of aldosterone has been found in extract B of urine from normal men and women on ordinary diets, but extracts A and G from the same urine did not cause sodium retention(16,17). Renal clearance of inulin (18) was measured during three 30-minute periods on one control day and on the last day of sodium deprivation.

TABLE I. Bioassay of Urine Extract B.

Case	Diet	Day	Effect equivalent to DCA, μg	Urine dose, min.	Aldosterone, μg/day
1	Usual		−2.0 ± 2.5*	/ 33	—
			6.4 ± 2.3	/167	1.8 ± .7*
	Low sodium	2	7.1 ± 2.5	/ 33	10.3 ± 3.6
		6	7.7 ± 2.1	/ 33	11.2 ± 3.1
	Recovery	4	6.7 ± 2.3	/167	1.9 ± .7
2	Usual		10.3 ± 2.1	/167	3.0 ± .6
	Low sodium	3	8.7 ± 2.1	/ 33	12.7 ± 3.1
		5	10.5 ± 2.1	/ 33	15.3 ± 3.1
	Recovery	2	11.6 ± 2.1	/ 80	7.0 ± 1.3

* 95% confidence limits of bioassay.

Results in the two subjects are similar. Sodium-retaining activity in the aldosterone fraction of urine extract B rose sharply during the first 2 or 3 days of reduced sodium intake (Table I). The increase in aldosterone was mirrored by the fall in urine sodium (Fig. 1 and 2). When usual sodium intake was resumed, aldosterone output decreased to normal levels, while urine sodium rose gradually to the control level.

Extract A showed little increase in activity, but there was a significantly increased yield of aldosterone from one extract G during sodium deprivation (Fig. 2).

On the low-sodium diet, serum sodium concentration and body weight decreased moderately, while the hematocrit increased.

Insignificant changes were observed in renal clearance of inulin and creatinine and in the output of 17-ketosteroids and 17-hydroxycorticoids. Serum potassium concentration increased after 2 days of sodium restriction, but fell on later days. Potassium output showed small variations from day to day without a consistent trend.

These observations could be explained in the following way. During sodium deprivation, aldosterone output is increased in response to decreased sodium content, volume, or pressure of the plasma or extracellular fluid. Aldosterone exerts its characteristic action by increasing tubular reabsorption of sodium(19). The reduced excretion of sodium balances the low intake. Resumption of a larger intake of sodium allows restitution of the lost sodium and water, at which time, the output of aldosterone returns to normal.

The increase in aldosterone during sodium deprivation is not accompanied by increased 17-ketosteroids or 17-hydroxy-corticoids in the urine. The opposite result is observed after administration of corticotropin to normal men, when the 17-hydroxy-corticoids and 17-ketosteroids are markedly increased without significant change in aldosterone(20). Normal amounts of aldosterone have been found in the urine of patients with hypopituitarism (16). Thus, neither endogenous nor exogenous pituitary corticotrophin appears to regulate the secretion of aldosterone.

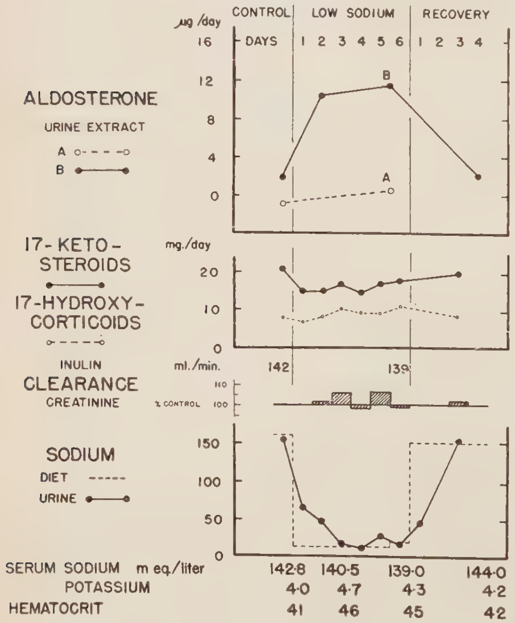

FIG. 1. Observations on healthy, 31-year-old man before, during, and after 6 days of sodium deprivation.

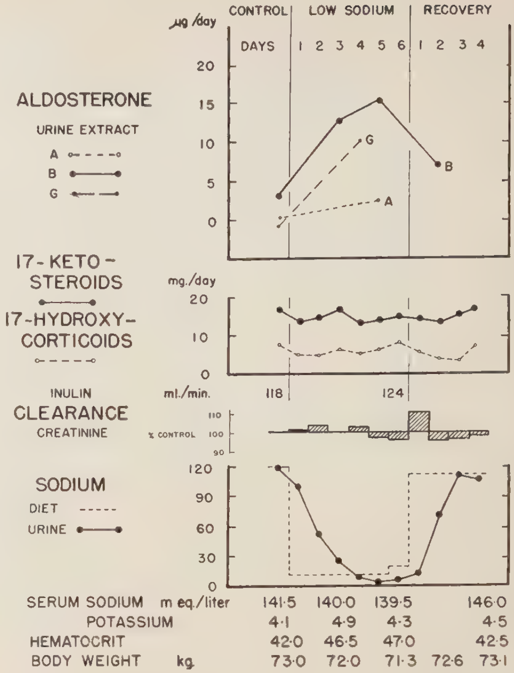

FIG. 2. Observations on healthy, 40-year-old man before, during, and after 6 days of sodium deprivation.

The insignificant variation in clearances of inulin and creatinine makes it improbable that glomerular filtration was affected under the conditions of these experiments. More severe sodium depletion might reduce glomerular filtration, but the effect of this reduction would be limited, since under the experimental conditions, only a small amount of sodium escaped reabsorption at a normal rate of glomerular filtration.

Summary. In 2 healthy men, restriction of sodium intake to 11 mEq./day was followed by increased aldosterone output, which mirrored the reduction of urine sodium. Insignificant changes were observed in renal clearance of inulin and endogenous creatinine and in the output of 17-hydroxy-corticoids and 17-ketosteroids.

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Sodium Retaining Material in Calf Adrenal Perfusates.* (21473)

FRANK UNGAR, GEORGE ROSENFELD,† EUGENIA ROSEMBERG, AND RALPH I. DORFMAN.
From Worcester Foundation for Experimental Biology, Shrewsbury, Mass., and Physiology Division, Naval Medical Research Institute, Bethesda, Md.

Simpson and co-workers(1,2) have isolated and identified what appears to be the most active adrenal steroid with respect to electrolyte metabolism. This steroid, aldosterone, has also been detected in human urines by Luetscher *et al.*(3) and Venning(4). In this communication evidence is presented for the presence in calf adrenal perfusates of a highly active sodium retaining material which may be aldosterone. The material was fractionated by paper chromatography and its activity established by bioassay.

Recent studies(5) have demonstrated that intact calf adrenals produced and secreted appreciable quantities of corticoids when perfused *in vitro* with a glucose-fortified, physiologically balanced and buffered salt solution of the following composition, in g/l: glucose 2, NaCl 8.2, KCl 0.20, CaCl 0.20, $MgSO_4$ 0.13, $NaHCO_3$ 0.60, NaH_2PO_4 0.05.

The artificial perfusion medium was gassed with a continuous stream of water-filtered 95% O_2/5% CO_2 and was supplemented with penicillin G (20,000 units/l) and streptomycin (0.1 gm/l). Employing these conditions, groups of 4 calf adrenals were perfused for 2 hours at 37.5°C in a multicycle system with one liter of fluid. At the end of each run, the adrenal perfusate was immediately frozen in a dry ice-acetone mixture and kept in this state until extracted for corticoids.

Methods. The thawed perfusates were combined and extracted 4 times with fresh portions of one-third volumes of redistilled ethyl acetate. The extract was dried with sodium sulfate, evaporated under vacuum, and fractionated by paper chromatographic procedures. The extract was first chromatographed for 72 hours in the toluene-propylene glycol system(6). The cortisone zone, which was separated from the cortisol, corticosterone and desoxycorticosterone zones, was rechromatographed, eluted with methanol (Zone A), and bioassayed for sodium retention. Zone A was then chromatographed in the benzene-ethyl acetate-methanol-water system of Bush(7) to

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† The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

TABLE I. Sodium-Retaining Activity of Calf Adrenal Perfusates (8 Adrenalectomized Rats per Group).

Bioassay No.	Material administered	Amt administered, μg	Mean urinary Na, mg/100 g/hr (range)	Desoxycorticosterone equivalent, μg
A	0	0	1.61 (1.31-1.90)	—
	Desoxycorticosterone	6	1.32 (.72-1.65)	—
		25	.89 (.48-1.14)	—
	Zone A†	24*	1.17 (.86-1.40)	9
B	0	0	1.86 (1.63-2.22)	
	Desoxycorticosterone	6	1.69 (1.31-2.64)	
		25	1.22 (.54-1.64)	
	Zone A-1‡	2*	1.58 (1.33-1.94)	8
C	0	0	1.26 (.90-1.66)	
	Desoxycorticosterone	6	1.07 (.56-1.54)	
		25	.64 (.55- .83)	
	Zone A-1‡	8*	.66 (.58- .73)	25
D	0	0	1.54 (1.43-1.72)	
	Desoxycorticosterone	6	1.65 (1.34-1.94)	
		25	.73 (.58- .89)	
	Zone A-1‡	8*	.99 (.67-1.17)	16

* Estimated by U.V. determination at 240 mu.

† Paper chromatographic zone which would contain cortisone, aldosterone plus other unknown substances.

‡ Paper chromatographic zone free of known active steroids which would contain aldosterone plus other unknown substances.

separate the cortisone from the zone which would contain aldosterone (Zone A-1). Zone A-1 absorbed in the ultraviolet and exhibited the mobility of aldosterone in the two systems employed. It showed a blue color with the tetrazolium reagent and an orange color with 2, 4-dinitrophenylhydrazine. Zone A-1 was eluted from paper and also bioassayed for sodium retention. The bioassay method, a modification of the one by Marcus *et al.*(8), employs the bilaterally-adrenalectomized male rat weighing between 135-150 g, a sodium chloride load of 45 mg/animal, and a 6-hour urine collection period. The urinary sodium was determined by flame photometry. Unknown materials were assayed in parallel with a solvent control group (20% ethanol) and 2 other groups which received desoxycorticosterone at 6 and 25 μg dosage levels. The results, expressed in terms of desoxycorticosterone equivalents, are presented in Table I.

Results. Sodium-retaining activity was found in each of 4 trials using 2 different materials (Table I). Zone A material contained

TABLE II. Output of Aldosterone-Like Material in Calf Adrenal Perfusates, 2 Experiments.

Exp. No.	No. of glands (g)	Aldosterone-like material (desoxycorticosterone equivalents)* Total µg	µg/g (tissue)/hr
1	20 (65)	900	7
2	48 (155)	3300	11

cortisone in addition to the electrolyte active material. However, under the bioassay conditions employed, cortisone does not cause sodium retention and actually would induce increased sodium excretion at a dosage of 6 µg or more. Zone A-1 material was a more highly purified fraction from which the known active steroids including cortisone had been removed. Amounts of 2 and 8 µg of this material estimated by ultraviolet absorption at 240 mg caused sodium retention. Although desoxycorticosterone was used as the standard for bioassay it is likely that the material under study is aldosterone. Experience in our laboratory indicates that aldosterone is approximately 30 times more active than desoxycorticosterone.

The calculated amounts of sodium retaining material in the perfusates are listed in Table II. The outputs calculated as desoxycorticosterone equivalents per gram of adrenal tissue per hour were 7 and 11 µg. In terms of aldosterone equivalents, these values are 0.23 and 0.37 µg, respectively.

Summary. By means of paper chromatography and bioassay a highly active material, which may be aldosterone, has been demonstrated in isolated calf adrenal perfusates. Bioassay indicates that the calf adrenal perfusate contains 0.3 µg of aldosterone equivalents per gram of tissue per hour.

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Characteristics of Poliomyelitis and Other Enteric Viruses Recovered in Tissue Culture from Healthy American Children.* (21474)

MANUEL RAMOS-ALVAREZ AND ALBERT B. SABIN.

From Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Cincinnati, O.

This study was undertaken to determine whether or not avirulent poliomyelitis viruses occur in nature. For this purpose it seemed best to use only healthy children who had no known contact with patients having clinically recognized poliomyelitis. The tissue culture technic was used not only because of its simplicity but also because it provided a means of obtaining poliomyelitis viruses without selection of those particles which would multiply preferentially in the nervous system of monkeys. In 1953, during the months of July and August, rectal swabs were collected from 1566 healthy children in Cincinnati and Xenia, Ohio. In addition to 5 strains of poliomyelitis virus and one strain of Coxsackie virus, a much larger number of hitherto unidentified cytopathogenic agents was recovered. The purpose of this communication is to describe some of the characteristics of these viruses.

Materials and methods. The children used in this study came from families in the lower income groups. There were several different categories: clinic patients, nearby summer camps attended by city children for short periods of time, and orphanages. Only those clinic patients were used who had no current, febrile infectious disease. Sterile cotton swabs were immersed in Hanks balanced salt solution containing 100 units of penicillin and 0.1 mg of streptomycin/ml, passed through the anal orifice into the rectum, turned around and immediately removed. Some of the swabs were slightly colored with fecal matter but most were not. The swabs were then put into lusteroid tubes containing 1 ml of Hanks solution with antibiotics and were stored in a deep freeze at –20°C. These specimens were stored for 5 to 8 months prior to use. After thawing, the cotton swabs were squeezed against the side of the tube to express as much fluid as possible. One ml of Hanks solution containing 2,000 units of penicillin and 2 mg of streptomycin/ml was added to each tube, and when necessary one or 2 drops of 4.4% solution of $NaHCO_3$ was added to bring the pH to about 8.0 as indicated by the phenol red in the solution. The tubes were then left at room temperature for 30 minutes and put in an ordinary refrigerator until transfer of the fluid to the tissue cultures. Most of the fluids were clear while some were opalescent or turbid. Roller tubes containing pieces of cynomolgus monkey kidney planted on a thin film of chicken plasma and a lactalbumin hydrolysate medium were prepared and used by a procedure previously described from this laboratory(1). The tubes were used when an adequate outgrowth of epithelial cells had developed on the glass and the medium was not changed. Each of 3 tubes received 0.2 ml of each specimen. All tubes were returned to the roller drums, where they either remained during the entire period of observation or occasionally were transferred to stationary racks after one or more days. The first microscopic examination was made in about 24 hours to detect evidence of cytotoxicity. A cytotoxic effect was observed with less than 1% of the specimens and when it occurred a blind passage was made after an incubation period of 7 days. Cytopathogenic effects of the virus type were not observed before 2 to 3 days and occasionally not before 10 to 13 days. Accordingly, the period of observation was approximately 2 weeks. The culture fluids were harvested 24 hours after the cytopathogenic effect was complete, and passaged to establish that the cytopathogenic agent was transmissible. Subsequent identification procedures were carried out either with the first or second passage culture fluids, except for a few strains which required several passages in tissue culture before they produced extensive or complete cytopathogenic effects. All cytopathogenic agents were first screened by a neutralization test using the 3 types of lyophilized standard poliomyelitis monkey antisera prepared by Wenner *et al.*(2) and distributed by the National Foundation for Infantile Paralysis. Tests in our own laboratory indicated that in a final dilution of 1:10 these sera neutralized at least 10^5TCD_{50} (50% tissue culture cytopathogenic doses) of the homotypic viruses. For our screening tests we mixed equal volumes of serum diluted 1:5 and culture fluid diluted 1:5. A mixture of all 3 types of serum, each contained in a final dilution of 1:5, was also used to cover the possibility that more than one type of poliomyelitis virus may be present in one culture fluid. Cytopathogenic agents which were specifically neutralized by one type of poliomyelitis antiserum were designated poliomyelitis viruses and further tested for their pathogenic effects in monkeys and mice. Cytopathogenic agents which were not neutralized by these antisera in tests with culture fluids diluted 1:5 were then titrated in tissue culture and retested in a dose of $100TCD_{50}$ with the same sera. No additional poliomyelitis viruses were picked up by this procedure. All cytopathogenic agents which failed to be neutralized by the poliomyelitis antisera were then tested in one- to 2-day-old mice, which received a subcutaneous injection of 0.1 ml of undiluted culture fluid containing between 10^4 to $10^{7.2}$ TCD_{50} of virus. Only one of the cytopathogenic agents was pathogenic for the newborn mice. The cytopathogenic agents which were neither poliomyelitis viruses nor pathogenic for newborn mice were further studied serologically. They were first tested in $100TCD_{50}$ doses with each of 5 antisera against the antigenically distinct "orphan viruses" (Grigg, Cornelis, Rende, Berad, Farouk) described and kindly supplied by Melnick(3) and 2 antisera against such viruses (Mack and Kentucky) described and kindly supplied by Steigman *et al.*(4). When the tests with all of these sera proved to be negative, antisera were prepared in rabbits against some of the strains recovered in this study. The rabbits were first given one ml of culture fluid intravenously and one ml distributed in 5 sites intracutaneously. Two additional intravenous injections of one ml daily were followed by a rest period of 4 days and 2 additional series of 3 daily intravenous injections separated by a 4-day rest period. Thirty-four days after the last dose another intravenous injection of 0.5 ml was given and the rabbits were bled out 7 days later or 58 days after the first dose. The potency of the rabbit antisera was determined by testing serial dilutions against 100TCD$_{50}$ of the viruses used for their preparation. All the other viruses were then tested in 100 TCD$_{50}$ doses with the rabbit sera in final dilutions of 1:10 or 1:100, depending on their potency.

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Results. Thirty-one cytopathogenic agents were recovered from the 1566 specimens. Five of these were poliomyelitis virus (3 were Type 2 and 2 were Type 3), one was pathogenic for newborn mice and subsequently identified as a Coxsackie group B virus, and 25 agents could be identified neither as poliomyelitis nor as newborn-mouse-pathogenic viruses (*e.g.* Coxsackie, herpes simplex). They were also serologically unrelated to the 5 antigenically distinct "orphan viruses" of Melnick(3) and the 2 viruses of Steigman *et al.*(4), which, with 2 exceptions, were recovered from patients with suspected nonparalytic poliomyelitis. The data shown in Table I indicate that both the poliomyelitis and the unknown enteric agents were recovered more frequently from children under 10 years of age. This difference is particularly striking for the nonpoliomyelitis enteric agents which were recovered from 5.2% of 154 children aged one to 4 years and from only .2% of 683 children aged 10 to 14 (the one Coxsackie virus was recovered from a 6-year-old child). The data in Table II indicate that among children of the same age group, the incidence was highest among those who came to the clinics and lowest among those who led a more secluded life in orphanages.

TABLE I. Recovery of Poliomyelitis and Other Viruses from Rectal Swabs of Healthy American Children.

Age group (yr)	No. of children tested	Poliomyelitis viruses recovered	Other viruses recovered
		%	%
1- 4	154	1 (.6)	8 (5.2)
5- 9	537	2 (.3)	14 (2.6)
10-14	683	2 (.2)	2 (.2)
15-17	154	0	0
Unknown	38	0	2 (3.4)
1-17	1566	5 (.3)	26 (1.6)

TABLE II. Incidence of Poliomyelitis and Other Enteric Viruses According to Type of Population.

Age group (yr)	Type of population	No. of children tested	Poliomyelitis viruses recovered	Other viruses recovered
			%	%
1- 4	Cl*	131	1 (.7)	8 (6.1)
	Ca	14	0	0
	O	9	0	0
5- 9	Cl	86	1 (1.1)	5 (5.8)
	Ca	187	1 (.5)	6 (3.2)
	O	264	0	3 (1.1)
10-14	Cl	8	0	0
	Ca	192	2 (1)	1 (.5)
	O	483	0	1 (.2)
15-17	Cl	0		
	Ca	0		
	O	154	0	0
1-17	Cl	225	2 (.9)	13 (5.7)
	Ca	388	3 (.7)	7 (1.8)
	O	915	0	4 (.4)

* Cl = Clinics; Ca = Camps; O = Orphanages.

The data in Table III indicate the potency and specificity of the rabbit antisera prepared with 3 of the 25 unidentified cytopathogenic agents. Prior to inoculation the rabbit sera had no neutralizing effect on these viruses. The fact that intracutaneous injection of large amounts of these agents produced no skin lesions in the rabbits, and that these animals survived the intravenous injections of large amounts of culture fluid also serves to eliminate B virus(5) from consideration in their

TABLE III. Potency and Specificity of Antisera Prepared in Rabbits against 3 Human Enteric Viruses.

Strain inoc.	TCD_{50}/ml culture fluid	Total amt inoc., ml	No. of inoculations	Rabbit No.	Antibody titer—50% serum dilution endpoint vs. 100 TCD_{50} indicated strain HE 1	HE 2	HE 3
HE 1, Wallace	$10^{8.2}$	10.5	10	872	20,000+	0	0
				873	20,000+	0	0
HE 2, Bryson	$10^{6.2}$	10.5	10	874	0	320	0
				875	0	320	0
HE 3, Hill	$10^{5.4}$	10.5	12	754	0	0	3,200

origin and identification. The data shown in Table IV indicate that 22 of the 25 unidentified agents belong to 3 distinct serologic types—8 Type 1, 11 Type 2, and 3 Type 3. It is furthermore of interest that human gamma globulin (prepared from large Red Cross pools), tested in a final dilution of 1:50, neutralized 100TCD_{50} of the HE 1 and HE 3 prototype strains and the 3 unclassified strains but not the HE 2 prototype strain. The 3 prototype strains were readily filtrable through gradocol membranes with an average pore diameter of 310 mμ. Their size has not been determined.

TABLE IV. Serologic Classification of 25 Human Enteric Viruses* by Means of 3 Prototype Antisera.

Strain of virus	TCD_{50}/ml of culture fluid	Effect of indicated antiserum vs. 100 TCD_{50} of virus HE 1	HE 2	HE 3	Serologic type
Wallace	$10^{7.7}$	0/3†	3/3	3/3	HE 1
Burton	"				
Mills	$10^{6.9}$				
Remer	$10^{7.2}$				
45 S.A.C.	$10^{7.7}$				
294 S.A.C.	"				
Spencer	$10^{7.2}$				
Witton	"				
Bryson, M.	$10^{6.7}$	3/3	0/3	3/3	HE 2
" , R. K.	$10^{6.2}$				
" , R. O.	"				
Collier	$10^{6.5}$				
Hallums	$10^{5.7}$				
Hartnes	$10^{6.2}$				
Hegger	"				
Maddox	"				
Servizi	$10^{6.7}$				
Setty	$10^{6.2}$				
Slone	$10^{5.2}$				
Hill	$10^{5.4}$	3/3	3/3	0/3	HE 3
Daniels	$10^{5.2}$				
Flack	$10^{4.2}$				
Lang	$10^{6.2}$	3/3	3/3	3/3	—
Cocrel	"				
Gregory	$10^{5.5}$				

* None of these viruses was pathogenic for newborn mice.

† Numerator = No. of tubes with cytopathogenic effect. Denominator = No. of tubes inoculated.

The data shown in Table V indicate that all 25 unidentified cytopathogenic agents do not behave similarly in tissue culture. All HE 1 and HE 2 strains produce a rapid and complete cytopathogenic effect which is indistinguishable from that produced by the poliomyelitis viruses. The HE 3 strains and the unclassified Gregory strain are somewhat slower-acting but produce a similar cytopathogenic picture. Two of the 3 unclassified strains (Cocrel and Lang) are not only slower-acting but the cytopathogenic effect is distinguishable from the poliomyelitis type of change by the fact that the degenerated epithelial cells appear differently and remain in grape-like clumps.

The one strain that was identified as a Coxsackie virus was slow-acting in the initial passages but produced a cytopathogenic effect indistinguishable from that of poliomyelitis virus. All subcutaneously inoculated newborn mice exhibited "CNS signs" in 4 to 5 days but none showed paralysis. In the second passage in newborn mice, all subcutaneously inoculated animals died in 4 to 5 days while only 8 of 10 intracerebrally inoculated animals died after an incubation period of 6 to 14 days. In the subcutaneous group extensive necrotic lesions were now found in the interscapular and prevertebral brown fat, but none was seen in the muscles, viscera (including pancreas), or CNS. In the intracerebrally inoculated

TABLE V. Difference in Rapidity of Cytopathogenic Effect on Monkey Kidney Epithelial Cells of 3 Prototype and 3 Unclassified Strains of Human Enteric Viruses.

Type of virus	Original swab	Days required for appearance of 50% or greater cytopathogenic change in each of 3 tubes inoculated with undiluted culture fluid of indicated passage level					
		1	2	3	4	5	6
HE 1	6, 6, 7	1, 1, 1					
HE 2	3, 3, 4	1, 1, 1					
HE 3	13, –, –	6, 9, –	4, 4, 8	2, 2, 2	2, 2, 2		
Cocrel —unclass.	13, 13, –	5, 5, 5	4, 4, 4	5, 5, 5	4, 4, 4	4, 4, 4	3, 3, 3
Lang — "	6, 6, –	6, 6, 6	7, 7, 7	6, 6, 6	4, 4, 4	5, 5, 5	4, 4, 4
Gregory— "	9, 11, 11	3, 3, 4	3, 3, 3	2, 2, 2			

newborn mice extensive necrotic and infiltrative lesions were found in the CNS above the spinal cord, but none was seen in the brown fat, muscles or viscera. Four-week-old mice inoculated intracerebrally or subcutaneously with the same material remained well. Doctor Gilbert Dalldorf very kindly identified this virus as belonging to the Coxsackie group B, type 4 (Yale-Texas 13 strain).

The 5 strains of poliomyelitis virus, in the form of monkey kidney culture fluid, were each inoculated into 4 or 5 cynomolgus monkeys intracerebrally and into 4 cynomolgus monkeys intraspinally. The results in each case conformed to the pattern of intracerebrally avirulent, spinal paralytogenic variant viruses(1,6). The details of these tests will be described separately along with other studies on the characteristics of experimentally segregated and naturally occurring poliomyelitis viruses which are intracerebrally avirulent, spinal variants for cynomolgus monkeys. The results of tests for mouse pathogenicity indicated that all 3 Type 2 poliomyelitis viruses were also intracerebrally avirulent, spinal variants for this species, while the 2 Type 3 strains were not pathogenic by either route.

The prototype strains of the 3 immunologically distinct types of poliomyelitis virus are all virulent for monkeys by the intracerebral route and all the other strains tested in the original poliomyelitis typing program (7,8) were, by virtue of the procedures used in their isolation, also intracerebrally virulent in monkeys. It appeared possible, therefore, that if other immunologic types of poliomyelitis virus existed in nature which are avirulent for monkeys by the intracerebral route they might have been missed in the original typing program. Accordingly, it was desirable to test several antigenically distinct strains of the unidentified agents which produced cytopathogenic changes in tissue culture indistinguishable from those of poliomyelitis virus, by intraspinal injection in cynomolgus monkeys. The monkeys were inoculated into the lumbar cord with .1 ml of the first or second monkey kidney passage culture fluids of the strains indicated in Table VI. None developed paralysis and no unequivocal poliomyelitis lesions were found in any of them. One of the 3 monkeys inoculated with $10^{6.2}TCD_{50}$ of the HE 1 (Wallace) strain died of an intercurrent enteric infection in 3 to 4 days. There were no lesions in the inoculated zone but $10^3TCD_{50}/g$ of lumbar cord were detected in tissue culture. The available data do not permit a conclusion as to whether this represents residual inoculated virus or low-grade multiplication. The remaining 2 monkeys, inoculated with this strain and sacrificed 6 weeks after spinal inoculation, showed the expected nonspecific lesions at the site of inoculation in the lumbar cord but one of them (6419) also had "granulomatous" or "old neuronophagic" infiltrative foci in the gray matter of 2 levels of the medulla and in the vicinity of the oculomotor nucleus in the midbrain. It is of interest that both of these monkeys developed neutralizing antibody within 22 days after spinal inoculation, and that the level of antibody in 6419 was as high after the single spinal inoculation as after the subsequent intravenous boosters (Table VI). Among the other 6 monkeys inoculated with 2 other strains, there was

TABLE VI. Antibody Response in Cynomolgus Monkeys after Single Intraspinal Inoculation and 2 Intravenous Booster Doses of 3 Human Enteric Viruses.

Type and strain of virus	TCD_{50}/ml culture fluid	Cynomolgus No.	Antibody titer vs. 100 TCD_{50} of homotypic virus: Before inoc.	22 days after spinal inj. of 0.1 ml	21 days after first intrav. dose*
HE 1, Wallace	$10^{7.2}$	6418	0	6	200
		6419	0	200	200
HE 2, Bryson	$10^{6.2}$	6421	0	0	100
		6422	0	16	63
		6423	0	0	32
HE 3, Collier	$10^{5.7}$	6424	0	0	32
		6425	0	32	3200+
		6426	0	0	4

* The 2 intrav. doses given 22 and 29 days respectively after spinal inoc. Each intrav. dose was 2 ml for monkeys 6418 and 6419, 1.5 ml for monkeys 6421-6423, and 1 ml for monkeys 6424-6426.

only one (6421) which exhibited slight focal, interstitial infiltration in the reticular substance of one level of the medulla. One monkey in each group developed neutralizing antibodies within 22 days after the single spinal inoculation. These observations permit no conclusion regarding the possible relationship of these agents to the poliomyelitis group of viruses. The development of antibody after a single spinal inoculation of .1 ml of culture fluid suggests, however, that multiplication occurred somewhere in these cynomolgus monkeys.

The HE 1 and HE 2 antisera produced in rabbits (Table III) and in monkeys (Table VI) were also tested for complement-fixing antibodies with antigens prepared from monkey kidney cultures of the 3 types of poliomyelitis virus through the kindness of Dr. W. McD. Hammon and Miss Gladys Sather. None of the 3 most potent monkey antisera fixed complement with the poliomyelitis antigens, while all rabbit sera did. It was shown, however, that these rabbit sera also developed C-F antibody for normal monkey kidney. These data also permit no conclusion regarding an antigenic relationship between these strains and the poliomyelitis group, because hyperimmune poliomyelitis monkey sera appear to be type-specific in the complement-fixation tests(2) while sera obtained from patients infected with only one type of poliomyelitis virus frequently show extensive C-F reactions with heterotypic antigens.

Discussion. The significant facts about the poliomyelitis viruses recovered from these healthy children during a nonepidemic year are that none of 5 strains were Type 1, the type recovered with greatest frequency during epidemic periods(3,7) and that all were of the intracerebrally avirulent variety for cynomolgus monkeys(6). Similar studies are now in progress on rectal swabs from about 1800 young Mexican children to determine whether Type 1 poliomyelitis viruses may be encountered with greater frequency in a population which is presumed to have a much higher rate of inapparent or unrecognized infection with all types of poliomyelitis viruses. The 25 other agents recovered from these children would appear to constitute a new group of enteric viruses. It is remarkable that none of them were antigenically related to the unclassified "orphan viruses" recovered by Melnick(3) and by Steigman *et al.*(4) from the stools of patients with suspected nonparalytic poliomyelitis. The fact that these new enteric viruses occur with much higher frequency in early childhood suggests that they are not normal inhabitants of the intestinal canal like the organisms of the coliform group of bacteria. The presence of antibodies in human gamma globulin for all but the HE 2 type of these viruses suggests that they cause widespread infection among human beings, and further studies are indicated to determine whether they are also responsible for clinically recognized disease.

Summary. Rectal swabs from 1566 healthy American children who had no contact with clinically recognized cases of poliomyelitis were tested in monkey kidney tissue cultures. Among the 31 cytopathogenic agents which were recovered, 5 were poliomyelitis virus (three Type 2 and two Type 3), one was a group B Coxsackie virus, and 25 belonged to a hitherto unidentified group of enteric viruses. This new group of human enteric or HE viruses is antigenically unrelated to the so-called "orphan viruses" recovered in tissue culture by Melnick and by Steigman *et al.*, from the stools of patients with suspected non-paralytic poliomyelitis. 22 of these 25 new viruses belong to 3 distinct serologic types (8 were Type 1, 11 Type 2 and 3 Type 3). The HE 1, HE 3 and the 3 unclassified strains were neutralized by human gamma globulin while the HE 2 virus was not. The HE 1 and HE 2 strains produced neither paralysis nor any significant lesions after intraspinal injection in cynomolgus monkeys. The 5 poliomyelitis viruses were all intracerebrally avirulent spinal variants for cynomolgus monkeys. The Type 2 poliomyelitis viruses were pathogenic for mice by the spinal but not the intracerebral routes, while the Type 3 viruses were avirulent for mice by both routes. Among children under 10 years of age who came to clinics from families of lower income groups, poliomyelitis virus was recovered from about 1% and the new enteric viruses from about 6%.

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Beta Irradiation Effects on Reticulo-Endothelial System Following Intravenous Injection of Radioactive Chromium Phosphate.* (21475)

ELEMER R. GABRIELI AND JOHN L. CUTLER.† (Introduced by C. N. H. Long.)
From the Department of Physiology, Yale University School of Medicine, New Haven, Conn.

Previous experiments in this laboratory indicated that in rats, small doses of X-rays as whole body irradiation induced large changes in the phagocytic functions of the reticulo-endothelial system (RES)(1). Since the entire animal was exposed to X-rays, these changes can be interpreted as a specific sensitivity of the RE elements toward ionizing radiation, or as a part of the general non-specific "stress" phenomenon, or both (Selye). To obtain further information concerning the radiosensitivity of the RE system, experiments were designed to reduce the non-specific and indirect effects of whole body irradiation by incorporating the radiating sources directly into the RE cells. This is practicable with negatively charged radioactive colloids because only the RE system removes and stores such matter. Therefore, the radiation originates entirely within RE cells.

In the pioneer investigations of Jones *et al.*, (2) colloidal radioactive chromium phosphate ($CrP^{32}O_4$) was used as a radiating source. Because most of the $CrP^{32}O_4$ is removed and stored in the v.Kupffer cells dispersed throughout the liver, this organ is exposed to an intensive radiation. Whereas Jones and co-workers studied tumor induction by radiation in the liver as a whole, the present investigation is concerned with the effect of radiation upon those host cells which phagocytized the radiating sources.

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† Present address: Tufts College Medical School, Boston, Mass.

Methods. Young adult male Wistar rats weighing 180-200 g were used. The colloidal suspension of $CrP^{32}O_4$ was prepared as previously reported(3). The radioactivity of these suspensions was determined by measuring the radioactivity in a unit volume under a Geiger-Mueller tube and comparing the counts obtained in unit time with a simulated P^{32} reference source (Tracerlab, Boston). The $CrP^{32}O_4$ suspension was injected into the saphenous vein under light ether anesthesia. Determination of the radioactivity content in various tissues(4) and radioautography(5) were carried out as previously reported. Parabiosis was performed using the technic of Bunster and Myer(6).

Results. Each of 20 rats was injected with 0.6 ml of a $CrP^{32}O_4$ suspension. The amount of P^{32} injected was determined to be 0.05 μc. During a 10 day observation period, no change in liver radioactivity (after correction for decay) was observed. This is in agreement with our previous results(4), indicating that there is no exchange between the P^{32} of the $CrP^{32}O_4$ and the P atoms of the body. Apparently the phagocytized particles remain within the RE cells without any release of P^{32}.

In a second series, 100 rats were divided into 4 equal groups. Each group was injected with 0.6 ml of a series of different $CrP^{32}O_4$ preparations, the only variable being their radioactivity content. One hour after the $CrP^{32}O_4$ injection, 5 animals from each group were sacrificed and served as a baseline ("initial distribution") for comparison with animals of the same group sacrificed at later times. The values obtained in the first 5 animals (*i.e.*, the values after 1 hour) were arbitrarily taken as 100%. The subsequent alterations were expressed as percentage deviation from the "initial distribution." Fig. 1 shows the results of the experiment. The radioactivity content of the liver in the group injected with 0.29 μc exhibited a statistically significant increase ($0.05>P>0.01$) the first 3 days after the injection, and a highly significant ($P<0.01$) increase by the 6th day. The increase in the liver radioactivity was still more striking in the group receiving 0.39 μc of $CrP^{32}O_4$. This increase was statistically highly significant ($P<0.01$) at each point. The same phenomenon, *i.e.*, increased radioactivity in the liver following the initial distribution, developed at a slower rate when $CrP^{32}O_4$ suspension contained 5.58 μc; and in the group injected with the largest amount of radioactivity (17.91 μc), no increase in the liver radioactivity above the initial distribution was noted throughout the observation period, although experiments to be cited later show that an active redistribution of $CrP^{32}O_4$ did occur, but apparently without net gain of radioactivity in the liver.

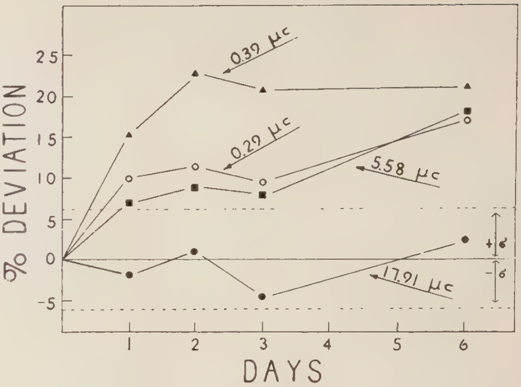

FIG. 1. Radioactivity content of livers of 4 groups of rats sacrificed at different intervals following i.v. inj. of 0.6 ml of radioactive chromium phosphate. Ordinate: % deviation from "initial" radioactivity in each group of livers (100%: radioactivity content of livers of rats sacrificed 60 min. after inj.). Abscissa: days elapsed after inj. Each point represents 5 animals. Dotted lines: stand. dev. in "initial" determinations. Figures at arrows indicate P^{32} content of administered $CrP^{32}O_4$ in each group.

The above experiments indicated that the distribution of $CrP^{32}O_4$ among the different organs is unchanged when the radioactivity content is very low (0.05 μc), while with greater P^{32} content of the $CrP^{32}O_4$, a "redistribution" takes place following the "initial distribution." In order to study the redistribution, another experiment was performed in which rats were injected with different graded $CrP^{32}O_4$ suspensions ("donor") and 24 hours later parabiosis was performed with an untreated litter-mate. Four days after the operation both animals were sacrificed and the livers were tested for radioactivity. Considerable radioactivity was found in the liver of

TABLE I. Radioactivity in Livers of Rats 4 Days after Parabiosis. One of the parabionts ("donor") was injected with $CrP^{32}O_4$ of various P^{32} contents before pairing with an uninjected one ("acceptor").

Total inj. radioactivity (μc)	Radioactivity in liver of donor (μc)	% of amt inj.	Radioactivity in liver of acceptor (μc)	% of amt inj. transferred
.045	.036	80.0	.0008	1.7
.055	.045	81.8	.0016	2.9
.082	.067	81.7	.0020	2.4
.082	.068	82.9	.0023	2.8
.082	.068	82.9	.0017	2.1
.135	.116	85.9	.0291	2.2
.135	.123	91.1	.0362	2.7
.450	.389	86.4	.0234	5.2
.450	.394	87.6	.0171	3.8
1.052	.862	81.9	.0493	4.7
1.052	.896	85.2	.0221	2.1
4.957	3.816	77.0	.3317	6.7
4.957	3.826	77.2	.4851	9.8
5.773	4.211	72.9	.4847	8.4
8.230	5.761	70.0	1.0863	13.2
10.277	6.470	63.0	1.0989	10.7
18.450	12.029	65.2	2.5092	13.6

the "acceptor" in each instance. Table I shows the results of these experiments.

The percentage of injected radioactivity found in the "acceptor" seems to increase with larger doses of P^{32} (*cf.* last column of Table I), indicating a higher rate of redistribution of the $CrP^{32}O_4$ in the "donor."

The distribution of radioactivity among the different organs of the "acceptor" paralleled that in the "donor," most of the radioactivity was in the liver in both animals, the spleen being the second most radioactive tissue. Besides the lung and the kidney, the other organs in the "acceptor" did not contain measurable amounts of radioactivity.

Organ	Counts/min. (× 10^3) Donor	Acceptor
Liver	39.47	1.75
Spleen	1.52	.07
Lungs	.31	.03
Heart	.11	—
Kidney	.14	.01
Abdominal muscle/g	.02	—

In previous studies(1), radioautography proved to be a very sensitive method of demonstrating radiation effects on the RE system. In the radioautogram of liver sections from intact "normal" animals a very characteristic distribution pattern has been observed—the particles are located peripherally near the interstitial septum between the lobules. This is because circulating colloidal matter first encounters a zone of v.Kupffer cells located around the edge of the lobule where it is efficiently phagocytized. (*cf.* Fig. 2 and 3). Fig. 4 is a radioautogram of a liver section from a rat injected with 0.05 microcurie 31 days before the second testing injection of $CrP^{32}O_4$. Two kinds of changes were observed in these animals; a. a considerable number of radioactive particles are located closer to the central vein, having penetrated the peripheral zone; b. large dense "sunbursts" are present at the periphery of the lobuli indicating that some v.Kupffer cells have accumulated a larger number of particles. Fig. 5 is a radioautogram from another rat, injected with 2.19 μc 6 days before the second testing injection. This picture was selected because changes similar to those appearing on this radioautogram were observed in all the other studies when the animals were injected from 0.5 up to 27.2 μc (highest dose used in these experiments). The large sunbursts failed to appear at these dose levels, the only change versus the normal pattern is a large number of particles located between the periphery and the central vein. It is difficult to quantitate changes in the radioautogram(5), but it appeared that with increasing amount of P^{32} in the $CrP^{32}O_4$ the efficiency of the peripheral v.Kupffer cells decreases.

Discussion. When negatively charged colloidal material is introduced into the blood stream, it will be removed and, if not digested, stored in the RE elements. This clearance mechanism of circulating colloids has been known since the discovery of the RE system. In the experiments described above, $CrP^{32}O_4$ was stored when the radioactivity content was low. However, when the radiation intensity was increased, the RE cells ceased to retain the $CrP^{32}O_4$ particles and released them back into the circulation. (The fact that the sites of uptake of $CrP^{32}O_4$ were identical in the "donor" and "acceptor" of the parabiotic pairs, shows that the redistribution proceeds through recirculation.) These particles were rephagocytized by other RE cells. Thus, fol-

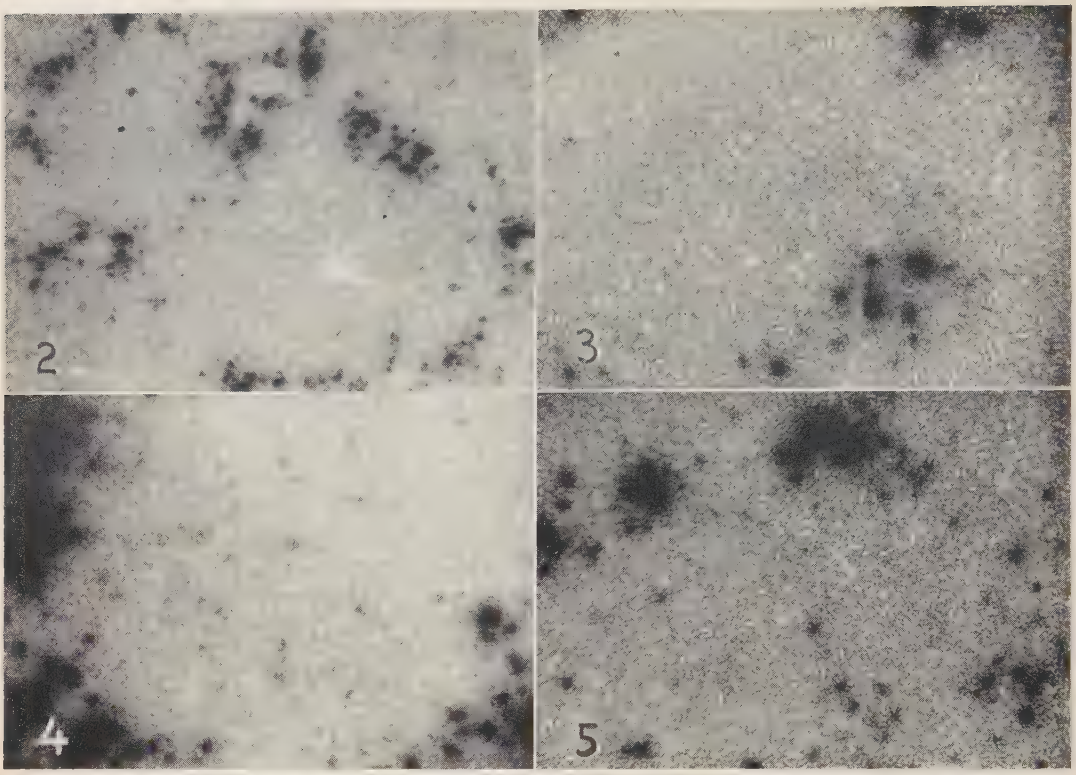

FIG. 2-5. Radioautograms of liver sections from rats inj. with identical amounts of the same CrP^{32}O$_4$ suspension 1 hr before sacrifice. Fig. 2 and 3: previously untreated rats. Note typical peripheral lobular distribution of radioactivity. Fig. 4: This rat received 0.05 μc of CrP^{32}O$_4$ 32 days prior to second "testing" CrP^{32}O$_4$ inj. Note large "sunbursts" (*cf.* text) on left and many radioactive particles in inner field of lobulus. Fig. 5: This rat received 2.19 μc of CrP^{32}O$_4$ 6 days prior to second "testing" inj. Note radioactive particles located inside peripheral zone, closer to central vein.

lowing the "initial distribution," a continuous redistribution took place. Obviously, the redistribution was a consequence of the increased radiation. Although the evidence is not conclusive, it is reasonable to assume, for example, from experiments with harmful agents (*i.e.* bacteria), that colloidal matter once removed by the RE system will be kept out of the circulation unless the RE system is damaged by the agent. Therefore, the observation that release of CrP^{32}O$_4$ increases with greater radiation intensity, is interpreted as increasing *radiation injury* of the RE cells.

Another complex mechanism is the *accumulation* of the radioactivity in the liver. The recirculating particles did not redistribute in the same proportion between hepatic and extra-hepatic RE cells, as during the "initial" distribution. This could be explained: a. by altered liver circulation, b. the liver possesses a greater phagocytic reserve than the extrahepatic part of the RE system. This latter fact has been observed following whole body X-irradiation(1). (Nevertheless, the first explanation cannot be ruled out completely.) Upon increasing the radiation dose to several microcuries, the accumulation during redistribution in the liver ceases to occur (cf. highest dose used). Redistribution proceeds vigorously in these animals, as demonstrated by parabiosis, but apparently the intensive radiation exhausts the reserves of the liver. This results in a redistribution without changing the ratios in the radioactivity of the different organs.

The radioautographic studies give further support to our above interpretation. The "sunbursts" (Fig. 4) are interpreted as a histological manifestation of the same phenomenon observed following the initial distribution; in animals injected with 0.29 and 0.39 μc there was an accumulation in the liver during the course of redistribution, and in the radioautogram the "sunbursts" indicate accumulation of particles in some v.Kupffer cells. Upon increasing the dose of P^{32} the accumulation in the liver and the "sunbursts" in the radioautogram ceased to occur. Although it is difficult to determine the degree and biological significance of the changes demonstrated by this technic, these studies seem to indicate an even higher radiosensitivity of the RE system than suspected from the changes in distribution (Fig. 1). The hypothesis of Pfuhl(7) and Toro(8) that the v.Kupffer cells at the periphery of the lobules are those which under physiological conditions play the role of phagocytosis (studied with India ink), while RE cells more centrally located along the sinusoids carry on digestion and storage, needs further confirmation. If this hypothesis is true, the radioautography (Fig. 4) following injection of 0.03 μc seems to indicate definite interference with this selective removal of the circulating colloidal matter at the periphery of the lobule.

Using $CrP^{32}O_4$, a considerable part of ionizing energy is absorbed by the cells surrounding the host cells. This is because of the long range of the beta rays emitted by P^{32} relative to the dimensions of the host cell. However, the amount of ionization occurring within the RE cells with a total dose of 0.29 μc was sufficient to interfere with their ability to retain the $CrP^{32}O_4$ particles. Using isotopes with softer emission, *i.e.*, S^{35}; C^{14}; H^{3}; etc., it would appear possible to destroy the RE system without appreciable damage to the surrounding tissues.

These results also confirm the opinion that the RE system is highly radiosensitive and that radioactive colloids should be used with extreme caution in humans(9).

Summary. Colloidal chromium phosphate suspensions containing graded amounts of P^{32} were injected intravenously into rats, thus exposing the reticulo-endothelial cells which phagocytized the radioactive colloidal matter to intensive beta radiation. 1. The radioactivity contents of livers were determined in rats sacrificed at different intervals from 1 hour to 10 days after chromium phosphate injection. The amount of radioactivity in the liver remained unchanged during observation period when chromium phosphate injection contained only 0.05 μc of P^{32}. Considerable increase in radioactivity of the liver due to redistribution within the body was observed following the 0.29 μc dose, and still greater accumulation of radioactivity obtained following 0.39 μc. When, however, 17.91 μc were injected, the liver ceased to accumulate radioactive particles. 2. Rats were injected with radioactive chromium phosphate a day before parabiotic pairing with untreated rats; 4 days later radioactivity was found in the livers of the uninjected partners. The portion of the injected amount which was transferred increased with greater P^{32} content of the chromium phosphate. 3. Radioautographic studies of liver showed changes similar to those following whole body X-irradiation, *i.e.*, distortion of the peripheral lobular pattern and large "sunbursts." The observed changes are interpreted as indicating great radiosensitivity of the RE system. It is suggested that destruction of this system by injection of isotopes with a shorter range of radiation than P^{32} could be achieved.

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